

27 November 1980

London medical schools stay in limbo

The University of London is making heavy weather of its attempts to reorganize its pattern of medical education. For most of the past year, the university but especially the medical schools which are constituent parts of it have been engaged in a fierce and frequently public argument about the proposals for rationalization put forward in February by a committee under Lord Flowers. Normally staid academics and, even more surprising, college principals with their origins in the civil service, have been writing to the newspapers, making public speeches and behaving as if this or that recommendation could be implemented only over their dead bodies. In one respect, at least, the volume (and the manner) of the protest has been effective. For the time being, nothing will be done. At its last meeting, the court of the university decided to postpone a decision until its next meeting, in February. This was a consequence of the decision of the senate of the university on 29 October (see *Nature*, 6 November) that no part of the proposed reorganization should be implemented without the consent of the medical schools concerned. So the hapless planning committees of the university are once more in session, seeking a formula that will win the consent of legally autonomous and temperamentally unruly medical schools to a measure of central government which is at once unpalatable and unfamiliar. If the issue is again postponed in February, the university will be running true to form. In the process, the foundations of its medical education will be further eroded.

Delay is of course a familiar response of academic institutions to the need for decision. The University of London has been grappling with problems of medical education since 1966, when the report of the Royal Commission on Medical Education, recognizing that the university is the chief source of qualified physicians in the United Kingdom, made specific proposals for reorganization. Since then, nothing much has happened, although the need that something should have become more urgent. The continuing decline of the population of London has reduced the numbers of patients to whom medical students in London have access; the National Health Service has embarked on a policy of redistributing hospitals and other medical services in accordance with these demographic trends and is now faced with a more permanent decision about the provision of hospital beds in London (brought about by the need either to replace old hospitals or to close them down). Although five among the twelve medical schools have been rehoused during this long period, resources available for teaching have steadily declined. Since 1973, the university has been trying to follow the advice of the University Grants Committee that more funds should be channelled to the medical schools. Its admission of failure provoked the Flowers inquiry. Things can only get worse while the university broods about its response. Given the importance of the university's medical schools in the pattern of medical education in the United Kingdom as a whole, the consequences of delay are not merely parochial. All medical schools will be affected.

The essential ingredient of the reorganizations proposed by the Royal Commission in 1966 and the Flowers committee in 1980 is the shotgun marriage. The Royal Commission had the twelve medical schools marrying off in pairs. In the event, one of these marriages was partly consummated in the relationship which has grown up between the London Hospital and St Bartholomew's Hospital (or, more accurately, the corresponding medical colleges). The other prescribed partners have fallen back jealously on their habitual celibacy, to which they have a legal right which they can surrender only voluntarily. The Flowers recommen-

dations took a similar form, but were inevitably more specific. Some marriages were to be arranged, two pre-clinical schools (at King's College and Westminster Hospital) were to be abandoned and the twelve postgraduate institutes within the university were either to be merged with teaching hospitals or to be relocated (in premises vacated by Middlesex Hospital). The Joint Planning Committee set up by the university senate and court acknowledges that the putative partners are within their rights when they insist that they would prefer to remain as they are. The university could nevertheless force change upon them by robbing them of funds — or by telling the National Health Service what future it sees for them. The effect of the senate's decision is that the pre-clinical schools at King's College and Westminster Hospital should be closed (and the clinical school at the latter merged with that at Charing Cross).

The university's dilemma is thus familiar to all those who work in universities in the process of change. Should a university administration force reorganization on unwilling academics? Should it, by failing to decide on a course of action, at the same time connive at a further deterioration in the quality of education? Or should it go all out for a decision in February, and if so which of the several canvassed options should it be? The greatest danger now is that the university will find itself plunging for one and sticking with it, come what may.

In reality, none of the schemes that have been advocated is ideal, although many of them contain common elements of agreed good sense. The shotgun marriages which have been proposed would often result in widely scattered groups of buildings united only by the new (and synthetic) names on their letterheads. What the university now needs is not a once and for all solution but a less ambitious and more persuasive programme of immediate action and a framework of management within which a more economical pattern of medical education can evolve, and be made to evolve, in the course of, say, a decade.

In the short run, there are administrative steps that should be taken — the rump of the Royal Free Hospital School of Medicine should, for example, be decanted from its old building, allowing the site to be sold. Similarly, the weaker among the postgraduate medical institutes — at a guess, six out of the twelve — should be looked at critically and perhaps even deprived of their academic pretensions. (Many of them would still have a useful service role within the National Health Service.) For the longer term, however, the university should be prepared to settle for a mechanism, not a prescription. And if the National Health Service demands some simple plan, it must be told that if the London medical schools are such an important part of British medical education, it is folly that their quality should be jeopardized by too hasty a solution.

In any case, the collective brooding about the future of the London medical schools has surprisingly left entirely out of account the paramount consideration — the character of the medical education the schools at present provide. The Flowers committee had some telling points to make about the way in which several of the existing schools are so short of resources that they cannot employ the full range of the academic staffs they need, but otherwise raised only the arid issue of whether it is better, within the peculiar pattern of British medical education, that intending physicians should be taken straight from secondary school and plunged into a hospital environment ("vertical integration") or, alternatively, allowed to spend their three pre-clinical years in a more normal type of college (the "multi-faculty" approach).



Nobody has seriously considered whether some quite different pattern, perhaps the American pattern, might be preferable, at least in some of the London medical schools. No serious consideration has been given by the university (as distinct from individual medical teachers or schools) to the pattern of the medical curriculum. The relationship between postgraduate education and postgraduate research is almost entirely unexplored. So is it not high time that the university that happens to be the most prolific source of physicians in the United Kingdom should ask for a stay of execution while these difficult but important issues are hammered out?

The obvious difficulty is that, as on many previous occasions, the medical schools and even the university would use delay as an opportunity for doing nothing. What can make them change their ways? One good sign is that the troubles of the past few months have given all concerned a nasty shock, which they will not quickly forget. But there are also obvious combinations of stick and carrot that could persuade the medical schools that it is in their common interest to find out what changed pattern would best suit the long-term needs of medical education. One present absurdity is that the twelve medical schools, nominally constituent parts of the same university, appear to be even more concerned with their independence than with the education of their students. The university has a committee to scrutinize

developments by the separate schools, but the joint planning board says it would be impossible for that body to arbitrate between the existing medical schools on questions such as which should do what.

Why ever not? Membership of even the loose federation called the University of London surely entails some sacrifice in a common cause. They should be willing, and if not should be compelled, to sacrifice some of their autonomy. The only basis on which they can continue to resist recurrent proposals of shotgun marriage is that they should be willing to join in a still more ambitious venture, one intended to weld the London medical schools into a single coherent complex of institutions concerned with the training of doctors. Whatever the separate schools now pretend, many of them are too weak to stand indefinitely on their own feet. Too many academic departments are too small to be viable. What the schools need is a system in which the university as a whole provides certain common academic services which are beyond their separate resources. Then they might have the best of both worlds — diversity of style but effectiveness (and efficiency) in their teaching. As things are, they invite only the further erosion of their resources, the continued impairment of the quality of their teaching and the loss of such sympathy as they retain from those who share the schools' own distrust of coercion in the administration of universities.

Best not to attend on Mr Moon

Next to Pope John Paul II and, possibly, the Reverend Ian Paisley, the internationally best-known religious leader is probably the Korean Sun Myung Moon, the founder of the organization called the Unification Church. In the past few years, the church has been constantly in hot water throughout the world, defending itself against complaints that in recruiting members to its ranks it frequently exerts undue influence on people who are temperamentally far from robust. The Unification Church is less well known (through its subsidiary organization, the International Cultural Foundation) as a sponsor of scientific conferences. The ninth "International Conference on the Unity of the Sciences" is now under way in Miami Beach (27–30 November), with the theme "Absolute values and the search for the peace of mankind". The chairman is the distinguished scholar Morton A. Kaplan from the University of Chicago. The vice-chairmen include Dr Frederick Seitz, most recently president of the Rockefeller University, and Dr J. S. von Euler, professor of medicine at the Karolinska Institute in Stockholm. This conference is as well blessed with distinction at the top as anybody can ask. The same, for what it is worth, is true of earlier conferences in the series — last time, for example, the chairman was Professor Eugene Wigner of Princeton. With such credentials, why should the question repeatedly be asked why people keep turning up?

The question is a controversial one. Those who participate in these conferences are usually at pains to emphasize that they have found them valuable, even stimulating. The same people are usually willing to keep on going back. The case for doing so was well put by Professor Nicholas Kurti two years ago, on the eve of the seventh conference held in Boston (see *Nature* 276, 206; 1978). The broadness of the themes of the conferences (in 1978 the subject was "the re-evaluation of existing values and the search for absolute values") does not always lead to the confusion that might be expected. Professor Kurti has said (and others have echoed him) that he has on the contrary found it useful to be required to think and talk about subjects that do not ordinarily come his way. Professor Kurti in his article made no secret of his view of the Unification Church ("many distasteful aspects") and of what he thinks of Mr Moon (not much), but he stoutly affirmed that the Unification Church could have derived no substantial propaganda value from the proceedings of the conferences, and that in any case no continuing obligation to the Unification Church would be incurred by accepting Mr Moon's hospitality (which is generous, to say the least). There is no reason to dispute

those opinions. It does not, unfortunately, follow that attendance is without consequence.

Quite apart from the source of their sponsorship, the conferences have some curiously shabby attributes. Perhaps the most serious complaint against them is that they are not so much sponsored as made possible by patronage. A part of the price paid by the assembly is that Mr Moon himself may choose to deliver an address of such vacuity that its thesis would not survive for ten minutes in an undergraduate seminar. By all accounts, however, Mr Moon's ideas survive more or less unscathed the gathering of Nobel prizewinners and other distinguished people who are required to listen to them. It is unseemly that scholars, but especially distinguished scholars, should behave in such a way. It is also unseemly that people should participate in conferences in which the participants, however individually distinguished, cannot collectively boast that they are the group ideally suited for the discussion of the broad themes with which they are faced. (To judge from the Miami Beach programme, some of the committee meetings, on the other hand, should be rewarding.)

Above all, it is unseemly that serious people should turn up at meetings where every other session title includes the word "values", where the title of the conference as a whole includes the phrase "absolute values" and where there is no means of knowing what these terms mean. It is true that participants are not required to assent to the "chairmen's affirmations" (read by Sir John Eccles to the fifth conference in 1976) that "scholars and scientists throughout the world should endeavour to search for absolute values and harmony among the sciences". Mr Moon might, however, get better value for his money if he required participants in advance to write a brief note of what they understand by these high-sounding words. As things are, participants are allowed to write on a subject of their own choice, receiving \$200 if the manuscript is sent in before 1 September and a further \$300 when what they write is "approved" for publication.

The case against these conferences, then, is self-contained. Professor Kurti may have been right two years ago to assert that participation does not necessarily give comfort (or much comfort) to the Unification Church, but the conferences themselves appear not to be as distinguished as those who participate in them, and should therefore be encouraged to wither away. For it is bad for the reputation of scholarship, dedicated as it is to the pursuit of excellence, that it should be seen to lend its name to extraneous causes. It matters less that they are those of the Unification Church than that they are second-rate.

Harvard finally backs off gene venture

Anxiety about conflict of interest wins

Harvard University's flirtation with the commercial exploitation of recombinant DNA research came to an end last week. After a period of agonizing and second thoughts by the faculty and administration (see *Nature* 20 November), the president of the university, Dr Derek Bok, put out a statement that the university would not after all participate in a company to exploit the work of Professor Mark Ptashne's laboratory.

What seems to have persuaded the university not to go ahead were the arguments of faculty members that a direct commercial interest by Harvard in the exploitation of ideas generated by one of its professors would create intolerable conflicts of interest. One argument was that the university would have found itself favouring the academics working for the commercial company in routine academic matters such as the appointment of staff, the allocation of laboratory space and even the provision of research funds.

It seems that the university administration has accepted these arguments only reluctantly. Mr Daniel Steiner, the university's Chief Counsel, said earlier this week that the university had approached Professor Ptashne as long ago as last February, suggesting the formation of a commercial company to exploit some of the research on which he was then engaged. It seems that until the faculty meeting in October, the university administration had not anticipated the strength of the reaction by some academics.

Other members of the faculty complain that the university administration was naive in its expectations of the commercial exploitation of recombinant DNA research. Under the proposed arrangements, the new company to be set up would have been financed by venture capital from commercial sources, would have had its own premises and its own managers and would not have been connected with Harvard by name. The university would have been given between 10 and 15 per cent of the equity without direct investment and there was also to have been a royalty on sales at a level not fixed.

In return, the university would have licensed the new company to use such patents as emerged only from Professor Ptashne's laboratory. Critics point out that the university would nevertheless have been a serious shareholder in the new company and that it could not have avoided committing resources of its own if,

for example, the new company ran into trouble.

Whether a commercial company based on Professor Ptashne's work will now be set up (without a shareholding by the university) remains to be seen. Professor Ptashne is thought to have been working since February this year, with the help of Professor T. Kanaguchi, on a novel method of making fibroblast interferon by cloning the gene at a more efficient expressing site in a plasmid in *Escherichia coli*. It is thought that the university has already committed some funds to this research.

Harvard's interest in the exploitation of patents arising from academic research dates back to 1975, when the university took advantage of a change in rules on patent law promulgated by the National Institutes of Health, and set up an office to encourage the patenting of inventions and the assignment of rights to the university.

Within the Harvard faculty, opinions remain divided. Some erstwhile supporters of the proposal regret the collapse of the administration's plan and hope that, in the

months ahead, less formal and perhaps more profitable ways of making progress may be possible.

Opponents, however, include some who argue that conflicts of interest are unavoidable if the university is to have a commercial interest in the exploitation of one professor's academic research. One irony that has emerged is that the university has indirectly acquired an interest in the recombinant DNA company Biogen, of which one of its professors, Dr W. A. Gilbert, is a prominent member. This interest has arisen independently, through a commercial investment in a venture capital company and in the normal process of university investment.

Among the critics, there are some who would use the events of the past few months as an occasion for a more general inquiry into the propriety of external entrepreneurial activities by any members of the faculty. One such critic said, earlier in the week, that Harvard was nevertheless comparatively "clean" — "You ought to look into what happens at MIT, down the river".

JET fusion experiment seeks growth

The case for making Europe's big fusion experiment, the joint European torus (JET), bigger and better — and twice as expensive — is now being argued by physicists connected with the project. They are arguing that the machine, now being built at Culham in Britain, should be equipped to allow full thermonuclear experiments using deuterium-tritium mixtures.

This proposal for "extended performance" is being discussed by the JET Council, the governing body for the project. It could be presented to energy ministers of the European Community in mid-1981, when the community's five-year fusion research programme is due for reappraisal.

"Extended performance" to reach ignition conditions was envisaged in the original JET design (completed in 1975) but was thought at the time to be financially too risky, for theorists had predicted that new high-temperature instabilities might set in before the plasma ignited. Since then, experiments in existing devices have led to much greater optimism. In particular, an experiment in the Princeton tokamak is taken to indicate that the feared instabilities do not exist.

This development makes the basic JET now look rather tame, and suggests that it should be possible to aim quite quickly at deuterium-tritium experiments. But this requires a greater power supply to increase the toroidal field power by 50 per cent and the heating power by 150 per cent, new diagnostic devices, remote handling

equipment and a degree of radiation protection (from neutrons emitted by the ignited plasma). There will also have to be facilities for storing and handling tritium (itself an expensive commodity).

The latest estimate for the cost of basic JET is 263 million European Units of Account (EUA) (£147 million), a figure largely explained by inflation since the original amount fixed in 1977 (185 million EUA). The cost of the extensions now proposed is not yet fixed, but is likely to double that amount.

Meanwhile, JET construction is going well, though building delays have made the mid-1983 physics target look tight. Twenty of the 36 toroidal coils have been delivered and tested successfully; and buildings are now either complete and occupied or well under way. The vacuum vessel is a little late — the first octant was due in October but is not now expected until January — but it is hoped to make up the time lost. Staffing is up to target: 300 out of 320 appointed.

Contracts have now been placed for almost all the major items for the basic machine but it will be necessary to place contracts soon for the sophisticated equipment needed for the extended version if the momentum is to be maintained. Under this programme, JET will be commissioned early in 1983, and will begin physics proper in the summer of that year. There would be two years of experiments with hydrogen, investigating scale effects, a year with deuterium, and then, if everything went smoothly, work with a deuterium-tritium mixture in late 1986. It

is not known how long JET could work with a radioactive plasma, until its materials become too active to handle efficiently.

The Princeton tokamak fusion test reactor (TFTR), which is of similar scale to JET, is already being installed in its buildings. According to JET officials, the TFTR will be running some six months before JET itself.

Robert Walgate

Telecommunications

Monopoly in doubt

Sir Keith Joseph, allegedly the British Cabinet's hard man on monetarism and other issues of grand policy, last week published a soft, even muddled, bill for the reorganization of the nationalized British telecommunications industry. The bill, which the government hopes will be law before the end of this session of parliament, would split the nationalized British Post Office into two parts, one (called the Post Office) concerned with mail and the other (British Telecommunications) with the development and operation of the telecommunications network.

The bill confirms British Telecommunications in its monopoly of the telecommunications services in Britain including — a minor surprise — the right to maintain all equipment connected to the network. But the bill provides for "approved" equipment to be attached to the network, for private organizations to use the network for selling what are called "value-added services" and even for letting the Secretary of State license private telecommunications networks if he thinks fit.

Although these developments were foreshadowed in Sir Keith Joseph's policy statement last July, the extent to which the bill leaves final decisions about the shading of the monopoly in the hands of the Department of Industry is surprising. Instead of attempting to define what technical criteria should be satisfied by privately supplied terminal equipment, for example, the bill gives the Secretary of State power to arrange for an approval procedure. Questions of when outsiders would be allowed to lease the telecommunications network (and at what cost) are being looked into by Professor Michael Beesley, but again it will be the Secretary of State who will decide what should be permitted.

British Telecommunications (which will not formally exist until the bill is law) is plainly unhappy with the extent to which the bill would give the Department of Industry a crucial and perhaps arbitrary say in its future business. Sir Keith's dilemma seems to be that, having shrunk from going the whole hog and defining British Telecommunications as a common carrier, he has had to fall back on ministerial direction as a way of nudging the corporation in his preferred direction.

The bill may thus be a recipe for constant wrangling between the communications network and the civil service (which, paradoxically, has been much involved with the affairs of all nationalized industries since the election of May 1979).

The new regime at British Telecommunications also promises continued uncertainty about the financing of the telecommunications network. The new corporation will be encouraged by the bill to set up new subsidiaries to compete with private manufacturers of terminal equipment, but will be required to finance these developments within the tight restrictions at present applied — and which, in effect, imply that most of the capital cost of renewing the British telecommunications network is paid for by current users of the network.

Last week, Sir Keith Joseph offered no escape from this corset except to the extent that British Telecommunications may be able to set up joint ventures with private industry which are financed privately and not under the corporation's financial control. Delphically, he declined last week to say what sorts of ventures he was thinking of, thus lending credence to the view at British Telecommunications that the proposed device will offer very little escape from the present squeeze.

Despite reports to the contrary, there appears to be no threat of interference with the programme of telecommunications research and development, based at the laboratories at Martlesham Heath in Suffolk. The government intends this bill to be law within a year. Its plan to sell off Cable and Wireless is the most likely snag.

Malaysian education

Pressures ease

Kuala Lumpur

Faced with internal racial pressures and a growing concern about academic quality, educational authorities in Malaya seem to be easing up slightly on the harshness of previous measures to reform the country's education system.

Two aspects of this policy have come under particular criticism. The first, known as restructuring, is the preference that has been given to Malay students and staff over those from Malaya's other two major ethnic groups, the Chinese and the Indians. The second, nationalization, has been the requirement that all school and university courses should eventually be taught in Malay rather than in English, both initially used as official languages after the country's independence from colonial rule in 1957.

Both goals are part of a new economic policy introduced by the Malay government in 1971. This followed widespread racial riots sparked off by Malay fears that their post-colonial political dominance was about to be challenged by the economically more powerful Chinese, who had voted

strongly for the main opposition party in the 1969 elections.

One result of the subsequent "reforms" is that, from 1985, all university courses will have to be taught in Malay, the culmination of a process which started in 1976 with the requirement that Malay be the language taught in primary schools, and which has been climbing the educational ladder one year at a time ever since.

The policy has been effective in increasing an awareness and use of Malay, now the official language in which all communications with civil servants, for example, must be carried out. But many university teachers now argue that an excessive concentration on Malay is already placing students at a disadvantage, particularly in science subjects where most textbooks and almost all scientific journals are written in English, and many scientific concepts have no Malay counterpart.

Partly in response to this criticism, the government is now boosting the teaching of English as a second language in Malaysian schools, arguing, for example, that English is necessary for graduates entering technical employment or intending to pursue postgraduate studies abroad.

A recent decline in the standard of English teaching in secondary schools was "alarming", said one education official last week, arguing that if it continued unchecked it would be a serious setback to the government's plans to increase the number of scientists and technologists on whom the country depended for its future.

A concern for educational standards has also prompted the government to relax slightly the constraints placed on foreign university staff — exceptions are now frequently made to the ruling that a non-Malaysian can only be given two consecutive three-year teaching appointments — as well as the strong preference given to Malays in awarding university places over Chinese and Indian students with equal academic achievement.

The latter relaxation has proved to be controversial, particularly as many Malays see positive discrimination in their favour as necessary to eliminate the dominance of the Chinese in many professional fields, including scientific research.

For example, opening a conference on the role of universities in the developing countries last week, the vice-chancellor of the University Kebangsaan Malaysia, Professor Datuk Awang Had Salleh, said that solutions based on groups rather than individuals remained an appropriate strategy for university entrance.

Erratum

The title of the article on the Indian environment, which appeared on page 207 of the 20 November issue of *Nature* should have been "Indian environment: Gandhi converted".

However, Malaya's Education Minister, Datuk Musa Hitam, is currently tightening up on the ease with which Malay students can at present enter university. In particular, he is reducing the proportion of Malay to Chinese and Indian students by 2 per cent a year until a final figure of 55 per cent *bumiputra* — literally "sons of the soil" — and 45 per cent non-*bumiputra* is achieved in the country's five universities. This is roughly equivalent to the racial balance in the country, but between 1970 and 1975, for example, the preference given to Malays increased their size as a proportion of student intake from 50 to 65 per cent.

Speaking at the opening of an educational building in Kuala Lumpur, Mr Datuk Musa was quoted as saying that this development should be taken as a "warning signal" to both *bumiputra* students and their parents that entrance to universities would in future depend less on their racial status and more on their academic achievement.

There is less political pressure to change the impact of present policies on the make-up of university staff. Part of the restructuring policy has been to increase considerably the number of Malay teachers and administrators in both schools and universities, again with the intention of redressing previous imbalances.

Chinese and Indian teachers accept both the logic of this policy and the political needs on which it is based. But in private, many express unhappiness that appointments, even at relatively senior administrative levels, are sometimes made with little respect for academic merit, and that in the process their own promotion prospects have been significantly reduced.

University staff in Malaya, however, are forbidden as government employees from taking part in any attempt to change policy. Lacking significant political power, their only options are frequently either to accept their reduced prospects or to seek teaching or research appointments abroad.

David Dickson

Antarctic research

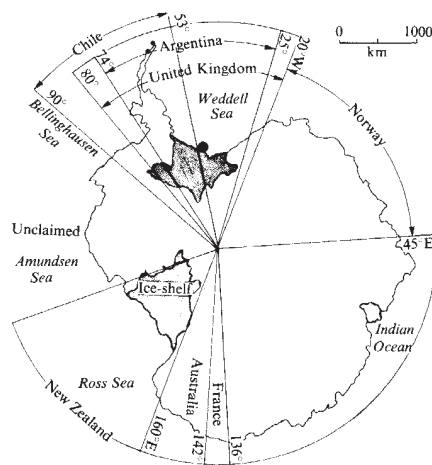
Germany joins in

A bid for a voice in the future development of the Antarctic may be behind West Germany's massive new research programme in the area. Details published recently* show that West Germany will become second only to the United States in the size of its investment in Antarctic research.

Although West Germany acceded to the Antarctic Treaty in 1979, it is still not admitted to the inner consultative group of countries which discuss matters beyond research, such as the exploitation of krill,

* *Antarktisforschungsprogramm der Bundesrepublik Deutschland*, Bundesministerium für Forschung und Technologie, Bonn. An English summary is available.

one of the last great untapped food resources of the sea, and the development of possible offshore oil supplies. In the wording of the treaty, admission to the consultative group is reserved for the original 12 signatories (which did not include West Germany) and for any later signatory demonstrating its interest in Antarctica either by conducting substantial scientific research activity there — such as the establishment of a scientific station — or by the despatch of a scientific expedition.



Germany for blob in Weddell Sea

West Germany is doing both. It is creating a new 20-man research and training institute at Bremerhaven to direct its Antarctic programme, building a supply and research ship and purchasing one or two Lockheed C130 transport aircraft (the largest planes on skis). Its commitment will cost DM 385 million (£85 million) to the end of 1983, with a large fraction going on capital equipment and buildings.

The Bremerhaven centre will be called the "Alfred Wegener Institut für Polarforschung" after the originator of the concept of continental drift, who died on an expedition to Greenland in 1930. The institute will open in January 1981 under the directorship of Dr Gotthilf Hempel, a marine biologist now at the University of Kiel, while the research ship, which has been placed on order at a German shipyard, will be launched in 1982.

Officials at the federal research ministry were at pains last week to demonstrate West Germany's long research interest in Antarctica. Germans were among the first explorers of the polar continent, with expeditions in 1873, 1901, 1911 and 1938. The last expedition, according to some commentators, was not research but an effort by Hitler to establish a territorial claim and was overtaken by the events of the Second World War. The region explored at that time (called New Schwabenland and Queen Maud Land) is now claimed by Norway. After the war, West Germany was too preoccupied with economic reconstruction to bother with the Antarctic. Interest revived in 1975 when a research ship was sent to investigate krill.

Antarctic politics

Antarctic politics are tortuous, a product of random historical and geographical factors and the current interest in food and oil resources. The Antarctic Treaty was set up after the International Geophysical Year of 1957–58, and ratified in 1961 by twelve nations: Argentina, Australia, Belgium, Chile, France, Japan, New Zealand, Norway, South Africa, the United Kingdom, the United States and USSR. Since then Bulgaria, Brazil, Czechoslovakia, Denmark, East Germany, West Germany, the Netherlands, Poland and Romania have acceded to the treaty, but of these only Poland has so far joined the all-important "consultative group" (which otherwise consists of the first 12 signatories). West Germany has applied to do so and is awaiting admittance.

The treaty forbids new territorial claims on Antarctica, but has no effect on previously asserted rights or claims. Before the treaty, Argentina, Australia, Chile, France, New Zealand, Norway and the United Kingdom had claimed sectors of the continent centred on the South Pole. These states recognize each other's claims, except Chile, Argentina and the United Kingdom, which have largely overlapping claims. The claims do not affect the placing of research stations: West Germany's proposed station will be on the edge of the Ronne ice shelf, near Berkener Island in the krill-rich Weddell Sea, on territory disputed between Argentina and the United Kingdom. The Antarctic Treaty confirms free access to all areas of Antarctica to all signatories. The legal status of the territorial claims, relevant only to resource development, is untested.

Surveys for an Antarctic research station began in 1979. The chosen site near Berkener Island is on the edge of the Ronne ice shelf, an extension of the Antarctic ice sheet over the shallows of the Weddell Sea. The station is now under construction and by late 1981 it will begin to serve as a scientific observatory for 45 scientists and technicians and a supply base for expeditions within a 1,000 km radius, wide enough to include the geologically interesting Palmer, Ellsworth, Pensacola and Shackleton mountain ranges.

Two other nations are also increasing their efforts in Antarctica — Australia and Argentina. Australia announced recently that it would completely re-equip its existing stations in Antarctica, at a cost of £20 million. Argentina is planning to construct the first all-weather, hard-surface landing strip as a potential refuelling base for commercial transpolar flights. Argentina is also constructing a new icebreaker for Antarctic work.

Robert Walgate

Bangladesh

Planning science

Dacca

Better use of the existing facilities and manpower for scientific research and development are given special attention in the Bangladesh government's Second Five-Year Plan, now published. In research and development, priority is given to agriculture, light and heavy industry, energy provision including nuclear power, housing and sanitation and transport.

The contribution of science and technology to the socio-economic development of the country has so far been relatively insignificant. Although Bangladesh boasts about 30,000 scientists and technologists with postgraduate qualifications, some 7,000 engineering graduates and more than 100,000 lower-level scientists, university research has often been "too academic" and in the government establishments research has often duplicated work done elsewhere. In nationalized industries there has been little initiative for innovative research, and in the private sector there has been virtually no research and development work.

Allocation for science and technology in the Bangladesh Second Five-Year Plan

	Estimated budget (in millions of taka*)
Agriculture	2,400
Industry	1,570
Flood control and water resources	750
Transport and communication	210
Health	390
Education	50
Natural resources	260
Nuclear power	1,000
Science, technology and research	1,550
Others	200
Total:	8,380

*US\$1 = 15.30 taka.

Main spending areas in the new plan (see table) are agriculture, industry, flood control and water resources, nuclear power plants and science and technology research. Of the allocation to science and technology research, nearly half of the 1,550 million takas budget goes to the Bangladesh Atomic Energy Commission. The bulk of the rest goes to the Bangladesh Council of Scientific and Industrial Research, the Space Research and Remote Sensing Organization and the Museum of Science and Technology.

The nuclear power programme includes a plan for the country's first research reactor for the production of radio-isotopes, a new 125-MW nuclear power plant in the western zone of the country and a study of the feasibility of extracting nuclear minerals from beach sand.

The space research organization is to concentrate on the completion of Bangladesh's advanced multipurpose

satellite ground station. The station's equipment is to be developed so that it can receive data from the various satellites including CMS (Japan), TROS-N and NOAT (United States) and METEOR (USSR). Important aspects of the satellite-based work for a developing country such as Bangladesh are a natural resources survey and a disaster-warning network. The five-year plan includes policy recommendations aimed at improving links between research centres and industry, improving primary and secondary education and giving further education a more practical bias. **M. Kabir**

Soviet fertilizers

Going it alone

Fertilizer production has been hived off from the Soviet Ministry of the Chemical Industry and now merits a ministry of its own, under Minister Aleksei Petrishchev, previously deputy chairman of the State Committee for Material and Technical Supply. At the same time, a new Minister of the Chemical Industry, Vladimir Listov, has been appointed. He was formerly deputy minister and has been working in the Party Central Committee since 1977.

These changes provide an indication of the direction of Soviet planning in the next five years, coming as they do in the closing weeks of the five-year plan. "Chemicization", as it is called, was launched by Khrushchev in 1958 and was continued by his successors. But Krushchev himself was less fond of chemical fertilizers than of the "virgin lands" — horizon-to-horizon grainfields in the erstwhile uncultivated steppelands of Asia. In 1965, his successors introduced a new agricultural strategy based on increased investment in agriculture, land improvement (drainage and irrigation) and an emphasis on intensive methods of cultivation, increasing the yield per hectare by the use of fertilizers rather than by expanding the total area cultivated.

The expansion of the chemical fertilizer industry was initially heavily dependent on Western technology, particularly the Pullman-Kellogg ammonia system and urea plants. Indeed, much of the capacity of these plants is still being used to pay off the cost of the equipment under compensation agreements.

Recently, however, the Soviet Union has been stressing its independence in all matters of chemical production. To some extent, this may be a reaction to world events. The main raw materials of the Soviet chemical industry are oil and natural gas, and the Gulf war has produced a spate of assurances that there is no oil shortage in the Soviet Union.

Poland is the Soviet Union's main supplier of another raw material vital to the fertilizer industry — sulphur. Political changes there may force Soviet planners to look more seriously at plans to utilize their

own relatively inaccessible sulphur beds.

Irrespective of politics, however, the Soviet fertilizer industry does seem to have reached a stage of relative technological independence. The new Chirchik ammonia plant, for example, is based on Russian technology and Russian contracting.

In spite of official emphasis on increasing the yield of food crops, Soviet agriculture's capacity for fertilizer remains far from satisfied. In 1976 only 50 per cent of the total grain cultivated area received any fertilizer at all.

The setting up of the new Ministry for the Production of Chemical Fertilizers strongly suggests that all branches of the fertilizer industry are to receive a boost in research and development. It also suggests that the forthcoming five-year plan will maintain the current emphasis on the "non-black-earth" regions in the west of the USSR, where the relatively poor soil demands considerable use of fertilizers.

If, as seems likely, the new ministry plans to build more fertilizer plants, the siting of these plants will be a major policy decision. At present, most plants lie in the non-black-earth zone, handy for the market, but far from Siberia, the main source of raw materials. Plants could, of course, be set up in Siberia, but the Siberian economy is in a bad way. **Vera Rich**

European plutonium

Project denied

Brussels

The future of the European Commission's support of research and development on plutonium cycling is in doubt. Last month, after a year-long search for a compromise, the Committee of Permanent Representatives (COREPER) rejected Euratom's second four-year plan even though this had been approved by the European Parliament last May. Officials now seem at a loss to know how an acceptable proposal can be devised.

The first programme ended in 1979 with what the commission described as "highly significant results" and encouraged it to draw up the more ambitious plan for 1980-84 now rejected. A total of 30 million European Units of Account (1 EUA = £0.56) was for research on the environmental effects of using plutonium (including the reprocessing of plutonium fuel), the transport of plutonium, the fabrication of mixed plutonium oxide and uranium oxide fuels and the recycling of used mixed-oxide fuels from light-water reactors.

France and the United Kingdom have led the opposition to the programme while Germany and Belgium have shown the greatest enthusiasm. The French and British are anxious to have cheap plutonium with which to fuel fast-breeder reactors and would rather buy plutonium from fellow member states than use recycled plutonium from their own reactors — the theory being that this would

increase the supply of plutonium and keep prices down.

Commercial rivalry is also said to have contributed to the rejection of the plan. The United Kingdom has already developed a process for fabricating mixed-oxide fuels and fears that a new research programme may compromise its capacity to collect royalties from the licensing of the process.

The desire to cut back on the commission's budget also probably contributed to the rejection of the programme, and thoughts are therefore now turning towards a "supplementary programme". Unlike a joint programme, this would be financed only by the interested countries.

Germany is committed to the plutonium-cycle programme because it wants to keep all options open, and public pressure at home makes any movement towards improving nuclear safety particularly attractive. Belgium, which generates 20 per cent of its electricity from nuclear power stations, feels that the programme would help towards securing steady fuel supplies. Italy and Holland seem prepared to go along with the programme but Ireland and Denmark are less sure — Ireland because it would draw little benefit in the foreseeable future and Denmark because it is interested only in the safety aspects of the plutonium cycle and would prefer the budget to be halved.

Jasper Becker

Project planning

Staged inquiries

The proposal that major planning issues of national importance, such as the fast-breeder reactor, should be decided in two stages, with a "project inquiry" followed by a local planning inquiry, was debated at a seminar on "The future of the big public inquiry" held at the Royal Institution, London on Monday 24 November.

The proposal has its origins in the work of a working party of the Council for Science and Society which issued a report in July 1979 recommending that project inquiries should investigate major proposals in depth, paying attention to the need for the project and considering all possible alternatives. One objective was to avoid lengthy and sterile wrangles at statutory local planning inquiries and even disruption by frustrated environmentalists.

There has recently been growing dissatisfaction with inquiries on national issues such as the building of major airports and motorways and nuclear installations. The siting of motorways seems to have been a particularly contentious subject and inquiries on such subjects have often been disrupted by highly regarded citizens.

The working party of the Council for Science and Society relied on the assistance

of Justice (the British section of the International Commission of Jurists) and the Outer Circle Policy Unit. The chairman was the barrister Paul Sieghart, vice-chairman of Justice, and the members were drawn from public administration, industry and planning.

At this week's meeting, Lord Kearton (with an extensive background of manufacturing industry) welcomed the proposal for preliminary project inquiries. He said few "big" inquiries were likely to arise in the near future and these would consist of proposals by the government for new motorways or for nuclear installations or refineries from bodies concerned with energy.

Sir Wilfred Burns, chief planner at the Department of the Environment, however, attacked the notion of the project inquiry. He argued that such a process would bring into question decisions reached democratically by parliament, the ultimate arbiter in matters of general policy.

The audience, consisting of officials, planners, consultants and members of pressure groups, seemed on the whole to have been in favour of the concept of a project inquiry. John Tyme, the veteran motorway protester, summed up the need for change by saying that many developers were "biased and ignorant, and many so-called experts on traffic or energy needs had been shown to be wrong".

Kenneth Mellanby

French science museum takes shape

Paris

The new French Museum of Science and Industry is at last beginning to take shape, even if at present only in the form of an architect's model. Two years after the commissioning of a report on the possibility of a new museum, and a year after that report was handed in, the final architectural plan has been selected. This pace, extraordinarily slow even for France, is a result of the problems attendant on a prestige project of this kind in a country in which "prestige" often equals "personal politics".

As originally conceived by the President of France, Valéry Giscard d'Estaing, the museum was to be the scientific equivalent, in grandeur and prestige, of the immensely successful Centre for the Arts created by his predecessor Georges Pompidou. To be erected on the 52-hectare site of La Villette in the north of Paris, the project was to be the largest, most modern science museum in the world. The original report, prepared by Maurice Lévy of the University of Paris, envisaged the conversion of an existing building on the site to a 100,000 square metre showplace of scientific and technical advance (*Nature*, 3 January).

Since then, however, a number of problems have surfaced which threaten to delay the project or to change it a great deal. There have been problems of money; the floor space available to the museum has

had to be reduced by more than half, to 40,000 m²; even so, the whole project is expected to cost 800 million francs — at a time of growing austerity in France. There have been clashes of personality; early in the year Lévy resigned as head of project, to be replaced after a three month interregnum by André Lebeau of the Conservatory of Arts and Crafts, an older, very traditional Paris museum.

Nor has the departure of Lévy ended all the differences of emphasis on policy questions among the museum staff. For example, the director of exhibitions, Goery Delacote, speaks of using the history of science and technology to "show the failures as well as the successes" so as to avoid a "triumphalist" conception of

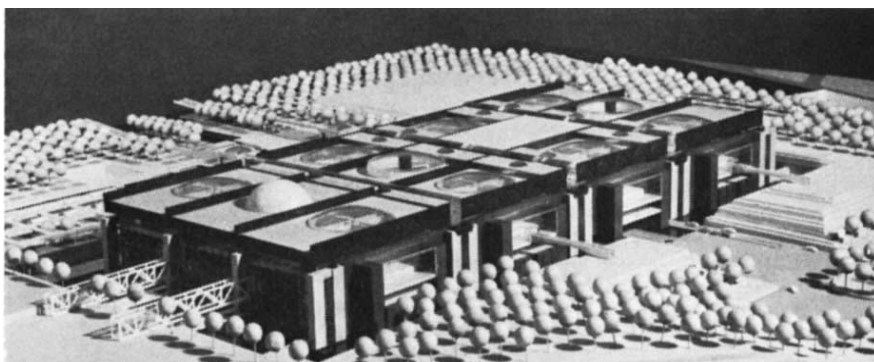
science, while general director Lebeau maintains that "the historical dimension should be present more to enlighten the comprehension of contemporary science than to tell the story of past science".

Added to all this is the fact that President Giscard d'Estaing retains a final say over all aspects of the museum's planning. Nor is this merely a formality. It was the president who chose the design of the winning architect Adrien Fainsilber from among the seven preselected by an international committee.

Thus the problems of a multiplicity of power centres, ambiguities in conception and increasingly tight financial resources suggest that the new museum is in for a prolonged and difficult gestation period which may last beyond 1984, the proposed date of completion.

Jim Ritter

Will Giscard, like Pompidou, have a centre...



CORRESPONDENCE

Medical education

SIR — In the article "Medical schools stay" (*Nature* 6 November, p.6) you say that King's College Hospital Medical School is reprieved. The Flowers Report did not in fact envisage the closure of King's College Hospital Medical School but suggested a fusion with Guy's Hospital, the product to be called the Lister Hospital. This idea has been replaced by a proposal that King's, Guy's and St Thomas's Medical Schools should form a consortium of equal partners under a common management body.

The debate in the University is now centred on the possible closure of the Medical Faculty of King's College London, from which the majority of our pre-clinical students come. The University has set up a subcommittee of its Joint Planning Committee called the Medical Costing Committee which is urgently to review pre-clinical education in London and to report its findings by January 1981. From its report the Senate and Court of London University will be able to make a decision on where medical students will derive their basic scientific training in London.

L. T. COTTON

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Risks at NRPB

SIR — Confidence in nuclear energy (*Nature* 19 June p.521) is not helped when individuals interested in the risks associated with it are inhibited either from expressing their opinions or from performing their work. The uninitiated who read the section on "Quality and integrity of the advisory and control authorities" in the Parker Report of the Windscale Enquiry would probably conclude that the record of the National Radiological Protection Board (NRPB) was clear in this respect, and moreover that although its late Director of Research, Dr G.W. Dolphin, in his generally censured paper on the Windscale workers, had underestimated radiation risks through a "mistake in methodology", the affair only showed that Dolphin was not infallible.

I have written (*New Scientist* letter, 13 January 1977) about the conditions at NRPB where my paper on radiation risks in the Japanese A-bomb survivors was held up by Dolphin for a year. One reason was his ignorance of statistics but he also objected strongly to risk estimates which were larger than he expected. The increases were not extraordinary but Dolphin's loyalties apparently remained with the Atomic Energy Authority (AEA) for he cautioned against making risk estimates which could be used against the fast breeder reactor. In contrast to the openly expressed bias supporting nuclear energy, there were no discussions about the possible risks from its development.

Before the merger forming the NRPB, the director of the Medical Research Council Radiological Protection Service publicly

warned staff, in the presence of the NRPB chairman and director elect, that the new management could not be trusted. Similarly, many of the AEA staff that joined the NRPB strongly distrusted the management who, they said, had abused their power in the AEA and exercised control over staff by, for example, making false reports on their work. However, they took care not to express any criticism in public and warned that it was futile to resist management decisions.

These management practices continued in the NRPB. Members of the staff found that it did not pay to disagree with the management who, in contrast, were apparently free to do as they pleased. When I objected to the management's duplicity and views on risks, the director of research warned that it would do me no good and became intent on making the conditions intolerable even at the expense of the work.

In 1977, soon after the Flowers Report made its criticisms of the NRPB, I wrote to Sir Edward Pochin, a member of the NRPB and an assessor at the Windscale Enquiry, concerning the working conditions at the Board but, although he promised to investigate, I received no reply. Recently my MP made enquiries and in the Board's answer it is claimed that Dolphin was the real author of the NRPB paper, published in my name, on risks in the A-bomb survivors, a statement which is clearly false from Dolphin's Windscale paper.

Evidently the regard shown to NRPB staff employed on similar projects depends primarily on who they are and not on the quality of their results. However, such anomalies do show the standard of the NRPB.

S.G. GOSS

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Reagan a plus?

SIR — It is with a mixture of incomprehension, dismay and disgust that we read David Dickson's assessment of the "plusses and minuses" of the 1980 United States elections (*Nature* 13 November, p.107), where Mr Dickson notes completion of the Clinch River Fast Breeder Reactor and resumed production and stockpiling of chemical warfare agents as expected plusses of the Reagan election. Since when is the development of a questionable technology with very likely adverse ramifications for the environment and for attempts to limit nuclear proliferation a positive direction in which to proceed? Since when is the development of new tools of murder and mayhem a plus for anyone, or does *Nature* — or at least its Washington editor — hold the view that science prospers when humanity is threatened?

MARK NOBLE

MICHAEL KLIMKOWSKY

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JACK PRICE

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Man's biosphere

SIR — I was sorry to see, in the unsigned editorial on Unesco (*Nature* 6 November, p.2), the passing reference to the Unesco "Man and the Biosphere Programme" as "chiefly valuable as sources of largely empty generalizations". Whoever wrote this editorial must have done so from a position of almost total ignorance of the MAB Programme. A research programme which has been endorsed by 79 countries, with more than 960 field projects, and involving more than 5,000 scientists is no empty generalization.

It is, of course, true that government departments and agencies in this country have shown relatively little interest once they have discovered that there is no "pot of gold" which can be readily tapped, as in the International Biological Programme. Nevertheless, scientists in research council institutes and in British universities have made, and are continuing to make, valuable contributions to the research of Third World countries in Asia, Africa and South America. They and their colleagues in these countries will be surprised, if not angry, at the glib dismissal of one of the most exciting and ambitious environmental research programmes as insignificant.

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Scientific warfare

SIR — R.A. Davis (*Nature* 6 November, p.8) has failed to notice that science and scientists do not exist in a social vacuum. Applications of technology generally raise questions concerning its impact on society and it is irresponsible for scientists to ignore these questions, especially when the technology concerned is to be applied to warfare.

One cannot help but be concerned when the US Army is interested in research directly related to chemical weapons, particularly since the recent go-ahead for the construction of a binary nerve gas plant. The United States is a signatory to several international agreements which specifically prohibit the use of these weapons — weapons which are indiscriminate in their effects upon noncombatants. To suggest that the development of an antidote will render these weapons useless is akin to suggesting that building fallout shelters makes the world safer from nuclear war.

It is incumbent on the participants of a democratic society to call attention to, and question policies which they do not believe serve the common good. When these participants are scientists and the policy concerns science then the pages of *Nature* are indeed the appropriate place to raise the issue.

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NEWS AND VIEWS

Sun-climate links

from T. M. L. Wigley

DOES the Sun vary in ways which could affect our climate and, if so, are these variations sufficiently large to have noticeable effects? The question of the Sun's influence on climate was examined in detail at a recent conference* where new data and recent advances in solar physics and Sun-climate links were presented.

If the Sun is to affect the Earth's climate, then some part of the Sun's output must vary. So far, the only variations observed seem unlikely to have a direct impact on climate. The solar 'constant' (that is the radiation output in the visible region, 300–400 nm, which accounts for 99 per cent of the Sun's energy flux) may not be a constant but evidence for its variability on annual and greater time scales is inconclusive. P. Foukal (Atmospheric and Environmental Research) reviewed this evidence and pointed out that changes have been observed directly only on shorter time scales; for example, those associated with the 27-day solar rotation period. The magnitude of these changes is extremely small, less than 0.1 per cent.

Longer-period and much larger-magnitude changes are well documented at shorter ultra-violet (UV) wavelengths. The magnitude of these fluctuations becomes larger as wavelength decreases, at least down to around 120 nm ($\text{Ly}\alpha$). D. Heath (NASA, Goddard Space Flight Centre) presented intriguing evidence that the direction of change over the past ten years has been different above and below 190–210 nm, with indications that these changes are associated with either the 11-year sunspot cycle, or the 22-year magnetic cycle.

Particle fluxes associated with the solar wind and recorded by their effect on the

Earth's magnetic field also follow the solar cycle. P. Simon and J. P. Legrand (Observatoire de Meudon and Laboratoire de Geophysique, EXTERNE-CNRS, Saint-Maure respectively) argued on physical and statistical grounds that the solar wind has two distinct components. The frequency of high-energy (10–100 MeV) proton events from solar flares and heliomagnetic disturbances, although the disturbances themselves are only short-lived (up to 100 min), varies approximately in phase with the sunspot cycle, while the lower-energy (around 1 MeV) particles emanating continuously from (mainly polar) coronal-hole regions show an out-of-phase relationship, the two combining to give a complex pattern of cyclic variations which is mirrored by variations in the Earth's magnetic field. These geomagnetic cycles have already been studied by Crooker *et al.* (*J. geophys. Res.* **82**, 1933, 1977) and by Feynmann and Crooker (*Nature* **275**, 626; 1978).

Just how UV and particle flux changes might affect climate is uncertain as direct effects, resulting from photochemical processes, are only observed in the stratosphere or above. We do not yet know how (or whether) these effects can be translated to the troposphere. Various mechanisms have been proposed (summarized by J. J. Berthelier, Laboratoire de Geophysique, EXTERNE-CNRS, Saint-Maure, and by A. P. Mitra, National Physical Laboratory, New Delhi) involving stratospheric aerosols, dynamic coupling between troposphere and stratosphere, changes in the Earth's electric field and/or direct radiative effects. These mechanisms are still speculative and require more detailed theoretical study and empirical testing; although the latter is hampered by the quality of currently available data. For example, although there is clear evidence that the solar proton event of 4 August 1972 affected stratospheric ozone, changes in ozone on longer (year-to-year) time scales have not yet been convincingly linked to solar activity (see Pittock *Rev.*

Geophys. & Space Phys. **16**, 400; 1968; Dutsch *J. atmos. Terr. Phys.* **41**, 771; 1979).

Statistical evidence of tropospheric changes associated with day-to-day solar variations was presented by J. M. Wilcox (Stanford University). Wilcox and his colleagues (see *J. atmos. Sci.* **33**, 1113; 1976) have found a relationship between the vorticity area index and the passage of solar magnetic sector boundaries, but the reality of this as a stable phenomenon has been questioned (Williams & Gerety *Nature* **275**, 200; 1978; Shapiro *J. atmos. Sci.* **36**, 1105; 1979). Russian work of a similar nature, reported by E. R. Mustel and co-workers (Astronomy Council of the Academy of Sciences of the USSR), apparently shows significant changes in surface pressure patterns 2 to 4 days after intense geomagnetic storms. Both of these phenomena are most evident in regions of large baroclinicity and in winter. It was suggested that this might imply some conditionally unstable initial condition in the atmosphere which is triggered in some way by a solar event, although recent modelling work by B. G. Hunt (*J. geophys. Res.* in the press) does not support this hypothesis. Further work is required before the reality of these short-term Sun-weather links is firmly established.

On the decadal time scale, tropospheric effects associated with the 11- or 22-year solar cycle remain controversial (see Pittock *Rev. Geophys. & Space Phys.* **16**, 400; 1978; *Nature* **283**, 605; 1980) and lack causal links. Interesting statistical relationships shown by R. G. Vines (CSIRO Division of Chemical Technology) and by C. J. E. Schuurmans (Royal Netherlands Meteorological Institute) (see also Schuurmans *Climatic Change* **1**, 231; 1978) point to a solar influence on the general circulation and may relate to the

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*'Sun and Climate', Toulouse, France, 30 September – 3 October was sponsored by the Centre National d'Etudes Spatiales, Centre National de la Recherche Scientifique and the Délégation Générale à la Recherche Scientifique et Technique. The proceedings of this conference will be published by the Centre National d'Etudes Spatiales as a companion volume to *Evolution of Planetary Atmospheres and Climatology of the Earth* (the proceedings of a conference in Nice in 1978; *Nature* **276**, 213; 1978).

recent solar-jet stream link found by Nastrom and Belmont (*J. geophys. Res.* **85**, 443; 1980).

On the century time scale, it was suggested long ago (de Vries *Koninkl. Ned. Akad. Wetenschap. Proc. B* **61**, 98; 1958) that changes in atmospheric ^{14}C levels might reflect changes in solar activity and climatic change. This idea was cautiously revived by Eddy (*Climatic Change* **1**, 173; 1977), and recent work by Stuiver and Quay (*Science* **207**, 11; 1980) has quite firmly linked changes in atmospheric ^{14}C to solar wind changes. Atmospheric ^{14}C changes also run roughly parallel to the envelope of solar activity as measured by the mean number of sunspots over a sunspot cycle. The Maunder minimum (1645 to 1715), a period of reduced solar activity, was a time of high atmospheric ^{14}C . ^{14}C fluctuations like that seen during the Maunder minimum have occurred frequently in the past and further observations were presented by H. E. Suess (University of California, San Diego, see also *Endeavour* **4**, 113; 1980). We can thus be fairly certain that the Sun does vary on the 100-year time scale. Conclusive proof could come from measuring the minute concentrations of cosmogenic ^{10}Be found in ice cores, using accelerator spectrometry (G. M. Raisbeck and F. Yiou, Laboratoire Renais Bernes du Centre de Spectrometrie, Nucleaire). Although these isotopic (^{14}C and ^{10}Be) variations do not in themselves indicate changes in solar output in the visible part of the spectrum, there is some indirect evidence of such changes. Changes in the Sun's radius should be accompanied by changes in luminosity (Sofia *et al.* *Science* **204**, 1306; 1979) and

radius changes can be calculated from eclipse data. Using material from the eclipse of 3 May 1715 collected originally by Sir Edmund Halley, NASA scientist, S. Sofia (Goddard Space Flight Centre), suggested that the Sun was about half an arc second bigger (and thus brighter) in 1715 than today. However, Parkinson (*New Scientist* 24 April, p203, *Nature* in the press;) has also examined eighteenth century eclipse data and deduces no changes in solar radius.

The link between atmospheric ^{14}C activity and the Sun is through the solar wind, and does not yet encompass changes in the solar constant. A statistically significant link between ^{14}C and climate had not yet been established either, in spite of the rough coincidence in timing between the Maunder minimum and the Little Ice Age. Thus, as for shorter time scale solar fluctuations, the Sun-climate link on the century time scale is still not proven. Recent work by Stuiver (*Nature* **286**, 868, 1980) has revealed only a few, barely significant correlations between reconstructed variations in solar activity (based on atmospheric ^{14}C variations) and climate data over the past 1,000 years. Results presented by L. D. Williams (University of East Anglia), T. M. L. Wigley and P. M. Kelly extend the analysis to the past 4,000 years and to a wider range of proxy climate variables, but still fail to find any statistically significant relationships. The main problem now is with the climate data base. On the 100-year time scale, our knowledge of the details of climatic change over the past millennia is very sketchy. As more palaeoclimatic information becomes available, the picture of past climates

becomes more and more complex. Although many significant fluctuations, similar qualitatively if not in magnitude to the Little Ice Age, have clearly occurred in the past, it is also evident that the response to solar forcing would involve circulation changes which might lead to considerable differences in trend and timing of, for example, temperature changes from place to place.

Changes in the Sun and in the Earth's climate on time scales significantly greater than 100 years, while of less immediate importance to mankind, are nevertheless fundamental to the understanding of the workings of the Sun and the climate system. Since the first solar model of J. Homer Lane in 1869, and especially in the last decade, solar physics has progressed enormously (reviewed by I. W. Roxburgh, Queen Mary College, London). The faintness of the youthful Sun (with luminosity only about 70 per cent of today's), predicted by stellar evolution theories is now quite firmly established and has led to a greater understanding of the importance of changes in atmospheric composition in determining changes in climate on the 10⁹-year time scale (Owen *et al.* *Nature* **277**, 640; 1979). But, as pointed out by M. B. McElroy (Harvard University), this factor is equally important today because of the large anthropogenic input of CO_2 resulting from fossil-fuel burning.

On intermediate time scales, between the century time scale variations implied by ^{14}C data and the long-term stellar evolution trend in solar luminosity, the possibility of fluctuations in the Sun's output is speculative yet still important. J.-C. Duplessy and A. Berger (CNRS, Gif-sur-Yvette) reviewed respectively the observational and astronomical evidence for the Milankovitch theory, which relates the periodic glaciations of the past few million years to changes in Earth orbital parameters. Although the evidence for this theory is very strong, it is still difficult to explain how the small total changes in incoming radiation, which result from orbital changes, can produce such major climatic effects. The key lies in the nature of orbit-related changes in radiation input, which occur not in total input, but in the seasonal and latitudinal distribution of incoming radiation. Although the global average change in radiation is only 0.1% or less, local variations of more than 10% can occur and the effects of even small changes can be amplified by ice-albedo and other feedbacks.

A more significant problem with the Milankovitch theory is that the observational data available for the past 400,000 years shows the strongest periodicity of radiation at 100,000 years with lesser peaks at around 40,000 and 20,000 years (Hays *et al.* *Science* **194**, 1121; 1976), while the astronomical data implies that the 100,000-year peak should be relatively less prominent. Hays *et al.*



100 years ago

Herr V. Bergso, in a recent work, "Fra Mark og Skov," has given some interesting data in regard to the habits of the Tarentula, *Lycosa tarentula*, Latr., whose nests he has traced and examined on the Roman Campagna. He found that the nest, which was well rounded and smooth, was approached by a tunnel which, after running about a foot straight down below the surface of the ground, made a sudden short turn before it finally descended for about another foot into the spider's abode. The superstitious error of assuming that the bite of the animal induces an irresistible desire of dancing is due to the fact, that dancing having been originally employed as a remedy against the poison, which is believed to be eliminated by profuse perspiration, the action of the poison was confounded with the means of its eradication.

"We the undersigned, captain, officers, and crew of the barque *Pauline* (of London) of Liverpool, in the county of Lancaster, in the United Kingdom of Great Britain and Ireland, do solemnly and sincerely declare that on July 8,

1875, in lat. 5°3' S., long. 35° W., we observed three large sperm-whales, and one of them was gripped round the body with two turns of what appeared to have a length beyond the coils of about 30 feet, and its girth 8 or 9 feet. The serpent whirled its victim round and round for about fifteen minutes, and then suddenly dragged the whale to the bottom head first.

"GEORGE DREVAR, MASTER

"HORATIO THOMPSON

"JOHN HENDERSON LANDELLS

"OWEN BAKER

"WILLIAM LEWARN

"Again, on July 13, a similar serpent was seen about 200 yards off, shooting itself along the surface, head and neck being out of the water several feet. This was seen only by the captains and one ordinary seaman, whose signatures are affixed.

"A few moments after it was seen elevated some sixty feet perpendicularly in the air by the chief officer and the following able seamen, whose signatures are also affixed —

"HORATIO THOMPSON

"WILLIAM LEWARN

"And we make this solemn declaration, &c.

"Severally declared and subscribed at Liverpool aforesaid the 10th day of January, 1877, before

"T. S. RAFFLES, J.P. for Liverpool."

From *Nature* **23**, 25 November, 84, 1880.

suggested that this may be related to a non-linear response. Two different explanations were presented here. C. Nicolis (Institut d'Aeronomic Spatiale, Belgium) presented a very simple model which showed how internal feedbacks could amplify an otherwise weak 11- or 22-year sunspot-related signal. A very different model developed by J. Oerlemans and J. M. Bienfiet (Royal Netherlands Meteorological Institute; see also *Nature* 287, 430; 1980) also produces a 100,000-year periodicity in the climate record, but in this case resulting entirely from internal factors related to isostatic rebound. This interesting result may, paradoxically, support the

Milankovitch theory. The strength of the 100,000-year periodicity is not constant and almost vanishes about 10^6 years BP. This would be hard to understand if the cause were purely internal, but less so if climatic changes were the result of orbital forcing. The lack of a 100,000-year periodicity in the early climate record may be related to the fact that perennial sea-ice cover in the Arctic only began around 700,000 years ago (Margolis and Herman *Nature* 286, 145; 1980). However, it may well be that around 10^6 years BP, the orbital parameters themselves, if more accurately known, have no 100,000-year signal. □

deep in northern latitudes) or thinner blankets in persistent oceanic, montane or tundra conditions. In the tropics peat is normally located in badly drained, riverine and coastal flats but can also occur in wet, cool climates at high altitudes. Tropical peats are usually deeper (up to 20m or more have been recorded) and contain many more macro-remains of vegetation plus mineral matter than their temperate counterparts.

The value of peat as an alternative energy source has perhaps been under-assessed and a broad spectrum of work is now being undertaken to harness its potential more effectively. Canadian authorities estimate that there is more energy potential in their peat deposits than in their forests or their reserves of natural gas. The National Energy Board calculates Canadian peat reserves at 89 billion tons which is equivalent to 500 trillion cubic feet of natural gas. The peat reserves of the US are put at 13 billion tons and the US Department of Energy has recently allocated \$2.2 billion for the development of alternative fuels, including peat as a major component. In the USSR peat fuels no less than 76 large electric generating plants. The Republic of Ireland generates one-third of its electricity and one-fifth of its total energy requirement from peat. Finland is in a stage of rapidly advancing technology in this field and it is estimated by 1990 that up to 8 per cent of the national energy requirement will be met from peat. Peat consumption in Europe has reached a point where regional producers cannot meet the demand. In the United Kingdom,

Peat — a resource reassessed

from J. A. Taylor and R. T. Smith

REPORTS at the Sixth International Peat Conference* suggest a need for a fundamental reassessment of the world's peat resources in industrial, agricultural and environmental terms. The total area of the world's peatlands is now estimated to be about 500m ha, equivalent to an area over half that of the United States. Canada (150m ha, 36%) and the USSR (170m ha,

40%) together monopolize over three quarters of the world's peatlands but there are also extensive tropical peatlands in the lowlands of S. America, Central Africa and parts of SE Asia.

Peat is organic matter derived from the slow decay of plant remains by the action of bacteria and fungi. Decay is finally arrested in the absence of free oxygen and sunlight, normally under conditions of ground or soil waterlogging which may be aided by an accompanying wet and cool climate. Peat may thus accumulate in badly drained basins forming mires (up to 10-m

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Genes controlling cell proliferation

from Paul Nurse

AN attractive hypothesis for the control of eukaryotic cell proliferation is that it is regulated at a particular point during the G_1 period before the initiation of DNA replication. This G_1 control has been called the 'restriction point' in mammalian cells (Pardee, A. B. *Proc. natn. Acad. Sci. U.S.A.* 71, 1286; 1974) and 'start' in the budding yeast *Saccharomyces cerevisiae* (Hartwell, L. H. *Bact. Revs.* 38, 164; 1974; *J. Cell Biol.* 77, 627; 1978). Cells made quiescent in a variety of ways become blocked at this G_1 control point, and cells shifted to conditions favouring proliferation have to pass this point before they become committed to the mitotic cell cycle. The control has been investigated genetically in *S. cerevisiae* by Hartwell and his colleagues, who have identified a gene *cdc 28* whose function is required before 'start' can be traversed. These workers

have suggested that before a cell traverses 'start', it monitors various conditions such as cell size, nutrient level, and the presence of conditions promoting conjugation and sporulation. If these conditions are satisfactory for cell proliferation, the cell begins a new mitotic cell cycle, but if they are not, the cell either becomes quiescent and enters stationary phase, or differentiates and undergoes conjugation or sporulation.

A recent study that has extended this genetic analysis in *S. cerevisiae* is described by Sudbery, Goodey and Carter in the current issue of *Nature* (p 401). They have isolated a novel series of mutants defining two *whi* genes, which undergo bud emergence and cell division at a reduced cell size compared with wild type. The *whi 1* mutants show a reduced cell size both during exponential growth and in stationary phase, whilst the *whi 2* mutation has a major effect only in stationary phase. When these mutants are made quiescent by deprivation of nutrients, they do not enter

stationary phase by becoming blocked at 'start'. They stop increasing in mass but they continue to initiate new cell cycles and to undergo division. When they eventually do stop proliferating, they are of a reduced cell size and have a reduced cell viability compared with wild type, and are also blocked in later stages of the cell cycle than the normal block point 'start'. These mutants are clearly defective in the gene functions required for an orderly transition of cells from the proliferating state to the quiescent state.

It has been suggested that mammalian cells may become malignant if they lose their 'restriction point' control (Pardee op. cit.), and as a consequence cells fail to accumulate at the proper point in G_1 when shifted to conditions favouring quiescence. If this is the case, then the *whi* mutants may be analogous to some types of malignant cell lines. Therefore, the further study of the *whi* and *cdc* 'start' mutants may also have implications for the problem of malignancy in mammalian cells. □

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the peat cover is quite extensive but to date has been regarded as too thin, too variable, too remote (that is, in the uplands) and not economically harvestable — a situation that may change with the introduction of new technology.

The most costly and time consuming aspect of peat exploitation is the treatment of the peatland before harvesting and the initial winning and drying of the peat. The technology for these purposes already exists, thanks to forest industries which have already solved many of the problems of mire drainage. In northern lands the short harvesting season has led to much effort being put into mechanization and into improving techniques to remove the water from either sods or milled peat. In Sweden, a centrifugation and belt press process has been developed which can process 6 cubic metres of peat slurry per hour down to 50 percent dry substance (that is, water content reduced to 50 percent by weight). From Finland comes a method of de-watering peat — the wet carbonization process — which produces dry pellets of partly carbonized peat, perhaps the most marketable product of the peat industry. Milled peat production is becoming more adaptable to mechanization, and subsequent processing is far and away more prevalent than traditional sod peat cutting. However, Finnish experience suggests that on the local scale at modest production, sod peat enterprises are likely to remain the more cost-effective. Peat is a most inconvenient source of energy compared to gas or oil but various gasification and liquefaction schemes are currently being tried out and may lead to major improvements. In these schemes processing plants will be located on the peat lands themselves as they are usually at great distances from centres of energy consumption. Peat-burning power stations, operated mainly by briquette peats, are commercially successful in the Soviet Union, Finland and Ireland but because of the variable consistency and calorific value of the peat, such stations are at least 5 percent less efficient than coal-fired ones.

As well as its value as an energy source, there appear to be many potential uses of peat as a raw material. Successful attempts have been made to use peatland for sewage disposal, on account of its great absorption capacity and its effectiveness in sequestering metallic cations. Other uses of peat include its role in activated carbon manufacture as a catalyst when treated with heavy metals, as a source of pharmaceutical and curative preparations, as a possible starting point for fertilizer manufacture, as a soil conditioner and even as a food additive.

The revaluation of the commercial potential of peat raises the question of the

possible harmful effects of its exploitation. The vast areas of peat function as stores of carbon, Bramryd (University of Lund) estimating that the annual average global rate of peat accumulation is 0.7mm — equivalent to about 0.21×10^{15} gC. Clearly, large-scale exploitation of peat and, in northern latitudes, interference in the fragile tundra ecosystem could help to trigger changes in the CO₂ balance. Only 10 per cent of the world's middle-latitude mires, mostly in Europe, USSR, USA and Canada, remain untouched. Accessibility for peat extraction and agricultural and

forestry development has been their demise.

New surveying techniques of peatlands are involving remote sensing, satellite imagery and georadar, notably in Sweden (Ultiksen, Lund Institute of Technology) the US (Hagen & Meyer, Minnesota Department of Natural Resources) and Scotland (Robertson, Macaulay Institute of Soil Research, Aberdeen). Automated monitoring of peatlands will boost not only scientific research into mires but also, ironically, their commercial exploitation in 'non-renewable' fashion.

Photochemical conversion and storage of solar energy

from Professor Sir George Porter, FRS

PHOTOCHEMICAL decomposition of water into its elements is potentially of great importance. It is theoretically possible in sunlight with efficiencies exceeding 10 per cent, and there are several routes to success which seem tantalizingly close. Yet so far, no satisfactory and efficient system has been discovered. This is the stuff that photochemists' dreams are made of and it is not surprising that many of them are now as excited as the molecules which characterize their craft.

The Third International Conference on 'Photochemical Conversion and Storage of Solar Energy' brought together most of the active research workers in this field under the Colorado sun and appropriately close to the new Solar Energy Research Institute which sponsored the very successful and well organized conference on photochemical conversions held in September. One hundred and fifty papers were presented, either as plenary lectures or as posters, all of which were on view and open for discussion throughout the week — a most satisfactory arrangement.

It was clear from the beginning that the principal interest of the participants would be in the rapidly developing subject of the conversion of light into chemical potential by methods which, although inspired by the mechanisms used in natural photosynthesis, are entirely abiological or artificial or, if you must, 'biomimetic'. Dr Mary Archer's (University of Cambridge) review of photogalvanic cells for electrical-power generation was decidedly pessimistic and there have been no significant advances in the use of photochemical reactions for the storage and release of

thermal energy. On the other hand, liquid-junction photovoltaic cells are developing rapidly and appear quite promising.

The photochemical reaction which attracts most attention at present is the production of hydrogen and oxygen, by the visible-light photolysis of water. The methods by which this desirable objective might be achieved range from biological ones, which retain some part of the *in vivo* system, for example isolated chloroplasts, through completely homogeneous photochemical reactions in solution to photoelectrochemical cells with one or two semiconductor electrodes, of the type originally used by Fujishima and Honda.

The apparently large gap between the last two is now, however, bridged, first by the use of colloidal metals and semiconductor suspensions in solution and, second, by the use of 'photochemical diodes' which consist of 'wireless' particles in which the two electrodes, usually a metal and a semiconductor, are in direct contact in a single entity which may be microscopically small. There is now an almost continuous transition between these different approaches and much success is being achieved with photochemical electron-transfer reactions in solutions which contain suspensions of such catalytic particles.

One of the principal difficulties in bringing about the liberation of gaseous hydrogen and oxygen in electron-transfer reactions in solution has always been that hydrogen requires the accumulation of two electrons while oxygen requires the accumulation of four. In an electrochemical cell these processes occur

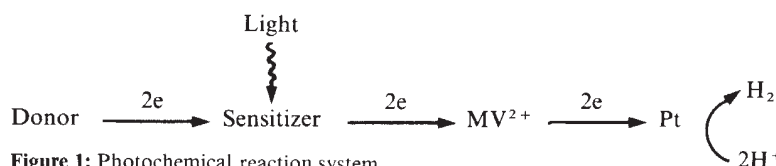


Figure 1: Photochemical reaction system

*The Sixth International Peat Congress was held in Duluth, Minnesota from August 17th to 23rd, 1980. Over 600 delegates from 25 countries attended, including, for the first time, scientists from China, Japan, New Zealand and Burundi.

at the electrodes and, if the overpotential is to be made as small as possible, platinum is usually preferred for hydrogen liberation at the cathode and ruthenium dioxide for oxygen liberation at the anode. The most important advance of the last few years has been the discovery that suspensions of these two substances will catalyse the evolution of hydrogen and oxygen in homogenous solution. Recent progress was reviewed by two of the principal contributors to this progress, Jean Marie Lehn (Strasbourg) and Michael Gratzel (Lausanne).

The natural photosynthetic system carries out the oxidation and reduction in separate steps, with a redox couple of quinone/hydroquinone between them, and most research has been concerned with one or other of these processes, using a sacrificial donor for the hydrogen elimination and a sacrificial acceptor for the oxygen liberation half-reactions. The water reduction reaction has been studied in many laboratories, nearly all of which use the system shown in Fig. 1.

MV^{2+} is methyl viologen (paraquat), which acts as electron carrier, and the sacrificial donor is usually EDTA, although many other substances which are irreversibly oxidized are equally satisfactory. As sensitizer, ruthenium

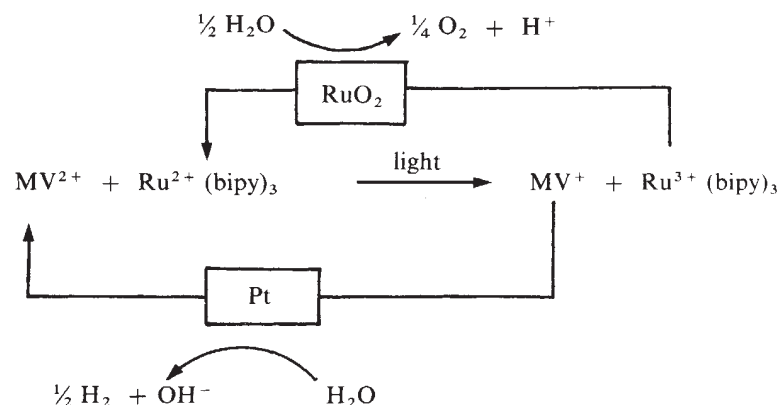


Figure 2: A four component system for the photochemical decomposition of water

trisbipyridyl has been most popular but water-soluble porphyrins have been used more recently and have many advantages; quantum yields of hydrogen production as high as 60 percent in green light were reported with zinc tetramethylpyridyl porphyrin as sensitizer.

The oxidation half-reaction, liberating oxygen, is in a less satisfactory state and, although ruthenium dioxide does act as a catalyst and liberates oxygen in the presence of an electron acceptor of sufficiently high redox potential, overall yields are low. Most surprisingly however, Gratzel and Kalyanasundaram reported oxidation and reduction with the simultaneous evolution of hydrogen and oxygen in a single system without sacrificial

electron donors or acceptors. The system was of four-components containing both catalysts, with methyl viologen as carrier and ruthenium trisbipyridyl as sensitizer (see Figure 2).

Of course, the liberation of hydrogen and oxygen together is not altogether desirable. Yields are low and the mechanism is not clearly understood; if confirmed it must obviously involve some specific kinetic selectivity of the redox components for the two catalysts.

In a highly competitive and developing field of research, preliminary reports which await confirmation are not uncommon. But this was a stimulating symposium in which everybody shared the excitement of a rapidly developing science.

Professor Sir George Porter, FRS, is Director of The Royal Institution

Far-infrared interstellar carbon

from a Correspondent

IN interstellar clouds, carbon is taken up partly in dust grains and partly in the gas phase, where it is distributed between ionized carbon C^+ , neutral carbon C and various molecules, mostly carbon monoxide with lesser amounts of other compounds such as HCN, H_2CO , HCO^+ , CH and CH_2 . Because CO is widely taken as a measure of the densities of clouds in which H_2 is the major constituent, the fraction of C existing as CO is a critical parameter in the determination of the cloud masses.

Observations of CO also provide information on the galactic isotopic ratio of ^{12}C and ^{13}C but, because of chemical exchange reactions, the relationship between ^{12}C and ^{13}C and the parent CO molecules depends, through a complicated chemical reaction scheme, on the density, temperature and age of the clouds. Information on the abundances of C and C^+ would thus help to make more certain the estimation of cloud masses.

Most of the radiant energy emitted by the gaseous component of interstellar clouds is contained in fine-structure transitions of C and C^+ and rotational transitions of CO. Emissions in the 1-0 and 2-1 rotational transitions of CO have been

measured at 1.3 and 2.6 millimeters, but it is the higher-lying transitions in the sub-millimeter region which contribute most of the cooling.

Recently the $^3P_1-^3P_0$ fine-structure transition of neutral carbon, the $^2P_{3/2}-^2P_{1/2}$ fine-structure transition of ionized carbon and the 6-5 rotational transition of CO have been detected from interstellar space. Laboratory measurements at the National Bureau of Standards using far-infrared laser magnetic resonance (R.J. Saykally and K.M. Evenson *Ap. J. Lett.* **238**, L107; 1980) established that the $^3P_1-^3P_0$ transition of ^{12}C has a frequency of 492.1623(7) GHz (and that the $^3P_2-^3P_1$ transition has a frequency of 809.3446(29) GHz). These accurate measurements made possible a search for and led to the discovery of the $^3P_1-^3P_0$ emission from several molecular clouds in the interstellar gas (T.G. Phillips, P.J. Huggins, T.B.H. Kuiper and R.E. Miller *Ap. J. Lett.* **238**, L103, 1980). The detection system used an InSb heterodyne bolometer receiver. The bandwidth is only 1 MHz and several observations were necessary to record a single Doppler-broadened spectral line.

A heterodyne system has also been used,

but with a GaAs mixer diode, to observe the 6-5 rotational transition of CO at 692.9495 GHz (P.F. Goldsmith, N.R. Erickson, H.R. Felterman, B.J. Clifton, D.D. Pack, P.E. Tannenwald, G.A. Koept, D. Bull and N. McAvoy, *Ap. J. Lett.* in the press) in emission from the Kleinman-Low plateau source in the Orion molecular cloud.

The $^2P_{3/2}-^2P_{1/2}$ transition of C^+ has a frequency of 1910 GHz and it has been detected towards the interstellar regions NGC 2024 and M42 (R.W. Russell, G. Melnick, G.E. Gull and M. Harwit *Ap. J. Lett.* in the press). The Cornell group used as a detector a stressed Ge:Ga photoconductor, cooled to 2K by pumping with liquid helium coolant, and a grating spectrometer (J.R. Houck and D. Ward, *Pub. A.S.P.* **91**, 140, 1979).

These papers demonstrate that a new region of the electromagnetic spectrum is accessible to observation. Sub-millimeter astronomy permits the direct observation of the major energy losses from the gaseous components of many regions of the interstellar gas and will provide powerful diagnostic probes of the physical environment in interstellar clouds where star formation is in progress.

Microbial degradation of xenobiotic compounds

from Philip R. Lehrbach

MOST organic compounds released into the environment are rapidly broken down by the activities of fungi and bacteria. However, certain man-made compounds (xenobiotics) are not quickly degraded. Public concern over the persistence of such compounds stems from their accumulation in food chains and their toxicity to living organisms. Xenobiotics in the form of pesticides, dye stuffs, surfactants and the waste products of industrial processes require detoxification before they are allowed to enter the environment and new techniques are needed to bring this about.

At a recent meeting* convened to discuss the capabilities of microbes and microbial communities to degrade exotic compounds, several strategies were considered from the viewpoints of industrial chemists, microbiologists and molecular biologists. H. Bretscher (Ciba-Geigy, Basel) discussed various methods for the total chemical and physical destruction of many xenobiotic compounds. Thermal degradation at incineration temperatures of 1,100°C, and the less developed but lower energy-consuming wet air oxidation (300°C) process were mentioned. But these processes have the disadvantages of high-energy expenditure and the total destruction of a potentially useful resource. In the future, economic constraints may dictate the use of biotechnical methods for the treatment of effluent. Such methods would be aimed at eliminating the environmental dangers while providing the basis for recovery of useful organic compounds used in the production of chemicals or microbial proteins.

Procedures are available (batch or continuous culture) for the isolation of bacteria or fungi able to grow on a particular organic compound. However, it is difficult to adapt these isolates so that they perform the same degradative function in the complex mixture of organic chemicals and salts present in industrial effluent. Reviewing the topic, W. Harder (Rijksuniversiteit Groningen, Haren) said that failure to isolate a pure culture able to utilize a particular compound may indicate that metabolism involves a community of bacteria and/or fungi. Important parameters such as culture conditions, interfaces, salt or acid gradients may have been overlooked. One example among many which illustrates the complexity of degradative processes is dichlorodiphenyltrichloroethane (DDT) metabolism. DDT is slowly broken down anaerobically to *o*-dichlorophenylmethane by

Aerobacter aerogenes. This compound is then co-metabolised by *Hydrogenomonas* to other less-toxic, chlorinated compounds.

One approach to the problem of waste disposal (D. Munneke, University of Oklahoma), which applies a detailed knowledge of enzyme technology but eliminates the frailty of the living organism, is to supply the appropriate inactivating enzyme to a contaminated area. Such an approach has been taken to detoxify contaminating amounts of the insecticide parathion from liquid waste effluent and industrial or domestic containers. In this case parathion is detoxified to *p*-nitrophenol in the presence of the enzyme hydroxylase. This enzyme can be used successfully in soluble (whole cell or partially purified) or immobilized (glass matrix) form. This example serves as a useful model for the development of other enzyme systems. However, several limitations are placed on this method of waste disposal. The enzymes used must be readily obtainable at relatively low cost, simple in their application particularly for domestic uses, stable, and not require an expensive cofactor to function. To justify this approach the chemicals to be inactivated must be of considerable environmental and public hazard (as was the case with parathion). The quantity and dispersal of the compound must be sufficient to require a concerted effort for its eradication.

U. Gasche (Cellulose Attishelz, Luterbach) and H. Kern (Institut für Botanik und Microbiologie, Jülich) focused on the complex problems of dealing with effluents from cellulose manufacturers. To achieve high yields of cellulose fibres, mechanical and chemical treatment of wood is necessary to convert lignin into physically and chemically altered derivatives which become a predominant by-product in the effluents. Lignosulphonates are lignin products formed in the acidic sulphite pulping process and are more resistant to microbial degradation than lignin itself. H. Kern discussed the possibility of enzymatic removal of the sulphonic acid groups and the subsequent bio-oxidation of the desulphonated products. He quoted data of Ban *et al.* (*Biotechnol. Bioengng* 21 1917; 1979) on mixed cultures containing yeasts and bacteria as an approach to economically feasible processing systems.

An understanding of the numerous biochemical pathways necessary for the bacterial degradation of various organic compounds is well advanced. Several participants suggested that this knowledge

could be used to develop bacteria with new metabolic capabilities. The reasoning is that bacteria able to degrade organic compounds that are structurally related to xenobiotics may be 'modified' to metabolize these new compounds. Modification using established mutation/-selection procedures or the specific genetic manipulation of appropriate genes determining catabolic enzymes to form new biochemical pathways were suggested. Such an approach has already been taken in the construction of haloaromatic-utilizing bacteria (H.-J. Knackmuss, Institut für Mikrobiologie, Göttingen, see *Nature* 277, 385; 1979). A similar approach was suggested from an appreciation of the metabolic diversity of methane-utilizing bacteria (methanotrophs) (I. J. Higgins, D. J. Best and R. C. Hammond *Nature* 286, 561; 1980).

Several important recent developments in molecular biology make this approach attractive to biotechnologists and may aid in the industrial exploitation of these organisms. First, the discovery that many catabolic pathways, including the degradation of substituted aromatic compounds (toluene, xylenes, naphthalene and styrene), are determined by the presence of transmissible plasmids. This makes possible the transfer of metabolic capabilities between various Gram-negative bacteria. Second, powerful new *in vivo* and *in vitro* techniques for the analysis and manipulation of genetic material permit rapid characterization of the structure and regulation of important degradative pathways, precise manipulation of the expression and the construction of multipathway organisms. The recent development of suitable vector systems in *Escherichia coli* and *Pseudomonas* (M. Bagdasarion and K. Timmis, Max-Planck Institut für Molekulare Genetik, Berlin) make this approach feasible.

The molecular analysis of the plasmid involved in the degradation of toluene and xylenes (the TOL plasmid) is probably the most advanced. F. H. C. Franklin and K. Timmis (Max-Planck Institut für Molekulare Genetik, Berlin) presented a functional map of the catabolic enzymes of the TOL plasmid based on the phenotypic properties of Tn5 (kanamycin resistance) insertion mutants. In this manner three genes, including the *xyIE* gene encoding 2, 3-oxygenase, were located. A subsequently cloned *XhoI* restriction fragment (*XhoI*-I) verified these data and showed expression of catechol 2, 3-oxygenase activity in the *Pseudomonas* host strain. These and similar studies on various catabolic plasmids will provide important information for the future construction of organisms capable of the specific degradation of xenobiotic compounds. □

*The 12th FEMS symposium on 'Microbial Degradation of Xenobiotic and Recalcitrant Compounds' held at Zürich, 15-19 September 1980. The proceedings will be published by Academic Press.

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ARTICLES

X-ray properties of quasars

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The X-ray properties of 111 catalogued quasars have been examined with the imaging proportional counter on board the Einstein Observatory. Thirty-five of the objects, of redshift between 0.064 and 3.53, were detected as X-ray sources. The 0.5–4.5-keV X-ray properties of these quasars are correlated with their optical and radio continuum properties and with their redshifts and variability characteristics. The X-ray luminosity of quasars tends to be highest for those objects which are bright in both the optical and radio regimes and which exhibit optically violent variability. These observations suggest that quasars should be divided into two classes on the basis of radio luminosities, spectra, evolution and underlying morphology and, thus, that quasars can make up a significant portion of the diffuse soft X-ray background only if the slope of the optical quasar log N –log S relation is steeper than 2 to $m_B \approx 21.5$.

As the most distant and luminous objects in the Universe, quasars have commanded considerable interest since their discovery nearly two decades ago. In the past few years, they have been surveyed by IR, UV, X-ray and γ -ray observers as well as optical and radio astronomers. X-ray emission from 3C273 was first recorded with a rocket-borne detector a decade ago¹, but before the launch of HEAO 2 on 13 November 1978, only four other quasars had been detected: MR2251-178 (ref. 2), 4UO241+622 (ref. 3), NRAO 140 (ref. 4) and NRAO 530 (ref. 4).

The first results of an Einstein Observatory quasar survey⁵, however, make it clear that a significant new window, the soft X-ray band, has been opened for the study of these objects. Thirty-two of the 40 sources in that survey were detected in the 0.5–4.5-keV range, with luminosities from 10^{43} to 10^{47} erg s⁻¹. These observations suggested that the summed contribution from the number of predicted optical quasars at faint magnitudes would exceed the diffuse soft X-ray background unless the optical quasar log N –log S relation is flattened significantly above $m_B = 20$, disagreeing with some optical measurements⁶. This surprising conclusion followed from the assumptions that: (1) the effect of the sample selection bias in favour of 'radio-bright' quasars was small, and (2) the effect of optical variability on the calculation of the X-ray-to-optical luminosity ratio could be ignored—simultaneous data at optical wavelengths were unavailable.

A second survey of quasars being carried out with the Einstein Observatory is reported here. Our sample consists of two components: a pointed survey in which 31 fields (one square degree each) containing 41 catalogued quasars were observed for an average of $\sim 2 \times 10^3$ s, and a serendipitous survey in which each of our other ~ 500 fields were examined for the presence of known quasars. The pointed survey sources were chosen to have a roughly uniform redshift distribution and to include many of the objects well studied in the radio and optical regimes. The 70 quasars in the other fields form a random collection of largely optically selected objects. Of this combined total of 111 quasars, 35 were detected as soft X-ray sources. For all of our observations, we have tried to obtain simultaneous optical measurements whenever possible but otherwise have tried to assess separately the contribution of the optically violent variables (OVVs) and to establish their optical-to-X-ray properties on the basis of nearly simultaneous measurements in the optical by A. Smith (personal communication). The initial results of this

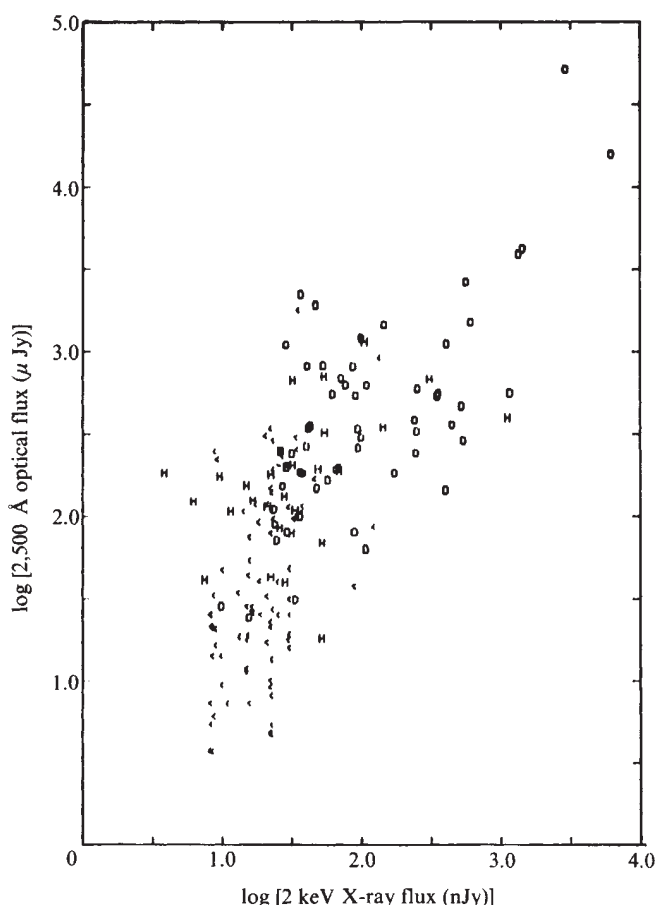


Fig. 1 Log of the flux at 2,500 Å (at the source) plotted against log of the flux at 2 keV (at the source). O, Objects with redshifts < 1; H, redshifts > 1; <, 3σ upper limits for objects. A clear positive correlation can be seen.

survey are reported, and their implications for quasar continuum emission mechanisms are considered. The significance of the revised values of X-ray brightness for the contribution of quasars to the diffuse soft X-ray background is also examined.

Observations

All of the quasar measurements discussed in this article were obtained with the imaging proportional counter (IPC) on board the Einstein Observatory⁷. Thirty-five quasars met the detection criteria of a counting rate $\geq 3\sigma$ above the background and coincidence within 80 arc s of the optical position (that is, within the 90% confidence X-ray error circle). For the stronger sources ($>5\sigma$), the positional coincidences were all better than 50 arc s. The common names of the 111 quasars examined, their positional designations, their redshifts z and the times of the observations are given in Table 1.

The IPC has sufficient time resolution to permit searches for flux variations down to time scales of milliseconds. Searches for flaring activity have been conducted for the 10 sources with counting rates >0.05 counts s^{-1} . No statistically significant variations ($>3\sigma$) were found on time scales of 10–1,000 s. However, several of the sources have been observed more than once several months apart. In some of these cases, large ($>100\%$) changes in the flux were recorded. These variability studies will be discussed elsewhere in conjunction with simultaneous observations obtained in other spectral regimes by ground-based observers.

As well as recording the arrival time and position of each photon, the IPC also provides crude spectral information. Although residual systematic uncertainties in the calibration of the IPC have so far prevented detailed analysis of the X-ray spectra, we believe that the fluxes calculated, following the

procedure suggested by Tananbaum *et al.*⁵, are correct to within 30%. The (0.5–4.5)/(1+z)-keV flux incident on the Galaxy, S_x , is used to calculate the 0.5–4.5-keV X-ray luminosity L_x (ref. 8):

$$L_x = S_x D \frac{4\pi c^2}{H_0^2} [z(1+0.5z)]^2 \quad (1)$$

where a Friedmann cosmology with $H_0 = 50$ km (s Mpc) $^{-1}$ and $q_0 = 0$ has been assumed. The logarithms of L_x and, where appropriate, the 3σ upper limits are given in column 5 of Table 1. We compare S_x and L_x to the optical S_0 and L_0 , calculated for the 3,000–6,000-Å band (at the source and corrected for galactic reddening⁹) using *UBV* magnitudes and a common energy index of 0.7, and to the radio S_r and L_r , calculated for the 3.1–8.1-GHz band (at the source) from published 1.4-GHz fluxes and spectral indices following the prescription given by Schmidt¹⁰. Where no radio flux has been measured, upper limits corresponding to the best available limiting fluxes are used. The spectral indices α_{r0} , from 5 GHz to 2,500 Å, and α_{0x} , from 2,500 Å to 2 keV, can then be found from

$$\alpha_{r0} = \frac{\log [L_r/L_0]}{5.380} + 0.595 \quad (2)$$

$$\alpha_{0x} = \frac{\log [L_0/L_x]}{2.605} + 1.200 \quad (3)$$

assuming power-law behaviour within each bandpass (see Table 1).

Table 1 Properties of 111 quasars examined with the imaging proportional counter on board the Einstein Observatory

Name*	Coordinates (1950.0)	Redshift z	Epoch (JD-2443000)	$\log [L_x(0.5-4.5 \text{ keV})]$ (erg s^{-1})†	α_{r0}	α_{0x}
UMT301‡	0100+0205	1§	1075.7	45.54	<0.56	1.32
PHL957	0100+1300	2.690	1052.3	<46.54	<-0.02	>1.62
PKS	0106+0119	2.170	1512.5	46.66	0.75	1.19
PKS	0109+1737	2.157	1077.0	<46.19	0.60	>1.44
PKS	0237-2322	2.263	897.7	47.16	0.70	1.29
NGC1073‡	0241+0109	0.599	1076.9	<44.41	<0.51	>1.43
NGC1073‡	0241+0108	1.945	1076.9	<45.75	<0.53	>1.31
NGC1073‡	0241+0107	1.411	1076.9	<45.35	0.60	>1.27
PKSO420	0420-0127	0.915	1099.8	46.12	0.70	1.16
NRAO190	0440-0023	0.844	1125.4	45.82	0.81	1.06
PKS	0736+0143	0.191	1167.0	44.44	0.58	1.53
4C05.34	0805+0441	2.877	1166.9	46.65	0.60	1.43
KP2¶	0805+0445	2.060	991.4	<46.23	<0.44	>1.19
MC5	0830+1115	0.589	1169.2	44.90	0.39	1.49
MC5	0830+1133	2.974	1169.2	<46.91	0.36	>1.35
KP¶	0847+1530	2.190	1169.9	<46.31	<0.37	>1.08
KP4¶	0847+1540	2.660	1169.9	<46.54	<0.22	>1.24
KP5¶	0847+1539	2.200	1169.9	<46.32	<0.37	>0.93
LB8755	0848+1533	2.010	1169.9	46.02	0.56	1.54
LB8796	0849+1530	1.320	1169.9	<45.67	<0.25	>1.43
OT1	0854+1910	0.331	1168.8	<44.23	<0.53	>1.53
LB8956	0854+1907	1.891	1168.8	<46.11	<0.36	>1.48
LB8991	0855+1846	1.013	1168.8	<45.36	<0.24	>1.52
LB9010	0856+1837	1.711	1168.8	<45.98	<0.32	>1.38
LB9029	0856+1855	1.286	1168.8	<45.63	<0.32	>1.47
0906+015	0906+0133	1.018	1013.1	45.85	0.68	1.31
NGC2859‡	0921+3444	2.250	1172.4	<46.17	<0.72	>1.20
NGC2859‡	0921+3444	1.460	1172.4	<45.61	<0.78	>1.13
NGC2859‡	0921+3444	0.230	1172.4	<43.72	<0.73	>1.30
TON 490	1011+2504	1.633	1017.2	46.60	0.39	1.55
NGC3384	1045+1253	1.111	1216.4	<45.59	<0.73	>1.14
NGC3384	1045+1253	1.107	1216.4	<45.59	<0.77	>1.06
NGC3384	1045+1253	1.131	1216.4	<45.61	<0.75	>1.09
NGC3384	1045+1253	1.134	1216.4	<45.62	<0.68	>1.24
NGC3384	1045+1253	1.192	1214.4	<45.67	<0.71	>1.17
NGC3384	1045+1253	1.280	1216.4	<45.75	<0.75	>1.08
NGC3384	1045+1253	0.520	1216.4	<44.80	<0.82	>0.98
NGC3384	1045+1253	0.497	1216.4	<44.75	<0.83	>0.96
WE1058W1	1058+7241	0.375	1152.3	44.61	0.68	1.34
3C273	1226+0220	0.158	1045.5	45.89	0.62	1.30
1246-057	1246-0542	2.212	1052.0	<45.93	<0.50	>1.70
3C277.1	1250+5650	0.321	1040.2	44.35	0.70	1.41
5C04.105	1258+2846	0.650	1064.7	45.06	0.57	1.42
5C04.127	1258+2837	1.373	1064.7	45.71	0.56	1.26

Table 1 contd.

Name*	Coordinates (1950.0)	Redshift z	Epoch (JD-2443000)	$\log [L_X(0.5-4.5 \text{ keV})]$ (erg s^{-1})†	α_{r0}	α_{0x}
W61972	1258+2839	1.922	1064.7	<46.26	<0.32	>1.39
B201	1257+3439	1.375	1221.7	<45.73	0.23	>1.59
B246	1258+3404	0.690	1221.7	<44.91	<0.15	>1.62
B471	1258+3422	0.774	1221.7	<45.06	<0.22	>1.40
KP31¶	1258+3432	2.010	1221.7	<46.21	<0.50	>1.19
KP32¶	1258+3417	1.800	1221.7	<46.05	<0.56	>1.03
KP33¶	1258+3416	1.930	1221.7	<46.11	0.51	>1.14
KP34¶	1258+3442	2.080	1221.7	<46.24	<0.34	>1.00
KP35¶	1258+3425	2.820	1221.7	<46.69	<0.38	>1.25
BSO6	1259+3427	1.956	1221.7	<46.13	<0.22	>1.46
KP36¶	1300+3433	2.880	1221.7	<46.73	<0.41	>1.17
KP37¶	1300+3427	1.700	1221.7	<45.97	<0.43	>1.16
KP38¶	1300+3421	1.800	1221.7	<46.05	<0.52	>1.02
W22722	1303+3050	1.770	1064.8	<46.25	<0.25	>1.34
W21541	1303+3123	2.047	1064.8	<46.44	<0.25	>1.35
1435+248	1435+2452	1.010	1065.3	45.45	0.69	1.20
OQ172	1442+1011	3.530	1077.2	47.06	0.68	1.41
NGC5866‡	1505+5557	0.706	879.7	<45.10	<0.56	>1.35
PKS	1510-0854	0.361	1089.0	45.30	0.67	1.29
KP57¶	1544+2112	2.050	1099.4	<46.22	<0.46	>0.91
KP58¶	1545+2054	1.850	1099.4	<46.08	<0.52	>0.91
KP59¶	1545+2100	1.890	1099.4	<46.11	<0.68	>1.17
3C323.1	1545+2101	0.264	1116.0	45.30	0.61	1.13
A2151*	1601+1747	1.430	1100.7	<45.61	<0.40	>1.43
A2151*	1601+1825	1.940	1100.7	<46.01	<0.47	>1.26
A2151*	1602+1753	3.020	1100.7	<46.61	<0.45	>1.24
A2151*	1603+1818	1.610	1100.7	<45.68	<0.51	>1.22
A2151*	1603+1755	1.770	1100.7	<45.87	0.69	>1.04
A2151*	1603+1806	2.060	1100.7	<46.05	<0.50	>1.20
A2151*	1604+1810	1.870	1100.7	<45.92	<0.54	>1.12
A2151*	1604+1739	2.010	1100.7	<46.02	<0.46	>1.27
A2151*	1604+1743	2.300	1100.7	<46.21	<0.42	>1.34
A2151*	1604+1756	2.740	1100.7	<46.47	<0.45	>1.25
A2151*	1605+1758	0.890	1100.7	<45.05	<0.45	>1.37
KP63	1604+2903	1.950	1258.0	<45.72	0.36	>1.36
KP64	1605+2851	1.800	1258.0	<45.61	<0.08	>1.04
KP65	1606+2910	0.060	1258.0	<42.25	<0.03	>1.10
KP66	1605+2902	2.000	1258.0	<45.75	<-0.07	>1.35
KP67	1606+2859	2.560	1258.0	<46.11	<0.04	>1.11
KP68	1607+2849	2.300	1258.0	<45.95	<-0.03	>1.25
KP69	1607+2903	0.350	1258.0	<43.85	<-0.02	>1.23
4C28.40	1606+2857	1.989	1258.0	45.70	0.69	1.43
TON 256	1612+2612	0.131	903.1	44.53	0.25	1.31
NAB1612	1612+2640	0.395	903.1	44.40	<0.24	1.52
KP70	1622+2648	2.100	1263.9	<45.82	<0.46	>1.10
KP71	1622+2656	3.160	1263.9	<46.45	<0.42	>1.16
KP72	1623+2709	2.400	1263.9	<46.01	<0.50	>1.32
KP73	1623+2700	2.300	1263.9	<45.95	<0.48	>1.03
KP74	1623+2654	1.900	1263.9	<45.68	<0.62	>1.15
4C26.48	1623+2657	0.779	1263.9	45.16	0.61	1.47
KP76	1623+2651	2.400	1263.9	<46.01	<0.33	>1.39
KP77	1623+2653	2.460	1263.9	45.91	<0.25	1.64
KP78	1623+2651	2.600	1263.9	<46.13	<0.13	>1.72
KP79	1624+2657	2.180	1263.9	<45.87	<0.36	>1.33
4C39.46	1632+3906	1.082	1101.0	<45.61	0.65	>1.34
4C38.41	1633+3814	1.814	1101.0	46.23	0.71	1.35
3C345	1641+3954	0.595	1112.6	45.96	0.73	1.22
KP90¶	1703+6051	1.980	1080.0	46.17	<0.55	1.49
3C351	1704+6048	0.371	879.5	44.92	0.55	1.56
PKS	1725+0429	0.293	1114.6	44.12	0.67	1.44
1729+501**	1729+5009	1.111	959.9	<45.77	0.64	>1.38
3C418	2037+5108	1.686	1019.4	46.12	0.81	1.30
OX 036	2121+0522	1.878	1190.8	46.45	0.63	1.38
PKS	2216-0350	0.901	1017.7	45.80	0.53	1.52
MR††	2251-1750	0.068	1017.2	44.49	-0.07	1.33
PKS	2251+1121	0.323	1406.4	<44.91	0.52	>1.49
PKS	2345-1647	0.600	1212.8	45.24	0.71	1.33

* Unless otherwise specified, data are from ref. 28.

† Systematic errors of $\pm 30\%$ (± 0.12 in logarithm) dominate all errors.‡ Quasar from Michigan-Tololo survey²⁹.

§ Redshift uncertain.

¶ Quasars near NGC galaxies³⁰.¶ Quasar candidates suggested by Sramek and Weedman^{31,32}.

* Quasars near Abell cluster 2151 discussed by A. A. Hoag (personal communication).

** QSO candidate from Jodrell Bank 966-MHz survey³³.

†† X-ray-selected quasar.

Data analysis

The data in Table 1 have been combined with data for 39 quasars previously discussed by Tananbaum *et al.*⁵ (excluding their data for 3C273, MR2251-178 and 3C351, which are included in our sample), two quasars claimed by Marscher *et al.*⁴ and 19 quasars discovered by Chanan *et al.*¹¹. The data were then analysed for possible correlations between X-ray behaviour and quasar continuum properties in other regions of the electromagnetic spectrum. The combined sample thus comprises 171 quasars of which 87 are positive detections. Twenty-six of these quasars were first discovered through X-ray observations. Sixty-five of the combined sample have a measured 1.4-GHz flux greater than 10 mJy. Of these 'radio-bright' quasars, 11 are classified as OVVs, 9 of which are known to be active¹².

A positive correlation was found between S_x and S_0 for the detected quasars. Although considerable scatter is evident in the correlation diagram (see Fig. 1), the correlation coefficient of 0.67 is significant at the 6.9σ level (using the Fisher z -statistic). Positive correlations also result from comparisons between S_x and the flux in the IR and at radio frequencies down to 15 GHz. Formally, however, there is no correlation between S_r and S_x for the sample of 65 quasars detected in both bands—the correlation coefficient is 0.04. This lack of correlation is largely attributable to three quasars: MKn205, PG0026+129 and MR2251-178. Nevertheless, Fig. 2, which is a plot of the detections and upper limits for all sources, shows a clear association between 'radio-quiet' quasars and a lack of X-ray emission. The fact that the X-ray and radio emissions are related can

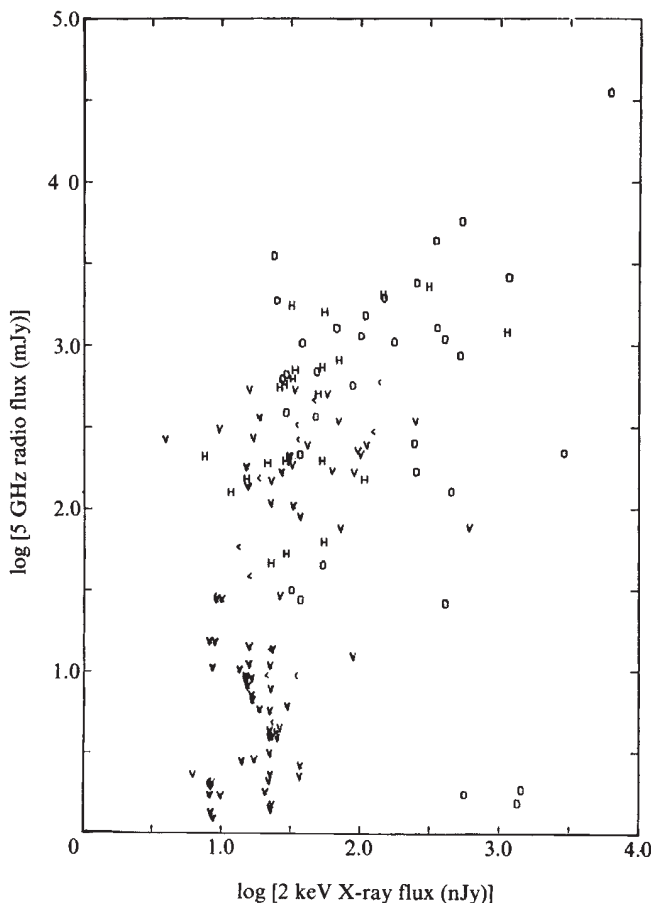


Fig. 2 Log of the flux at 5 GHz (at the source) plotted against log of the flux at 2 keV (at the source). Symbols as in Fig. 1. V, appropriate survey limiting fluxes for objects not detected in the radio. While the plot of detections forms a scatter diagram, the preponderance of X-ray upper limits corresponds to 'radio-quiet' objects in the lower left-hand corner of the diagram.

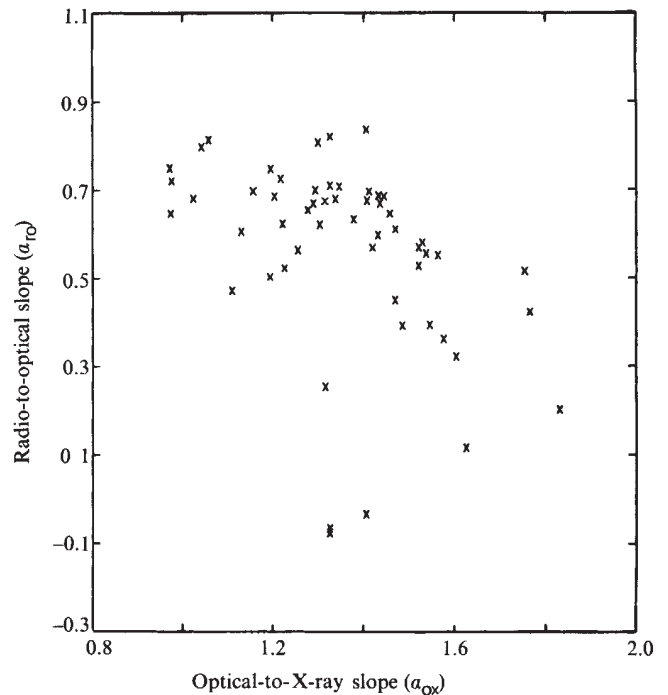


Fig. 3 A 'colour-colour' diagram of quasars. The radio-to-optical slopes for detected quasars are plotted against the optical-to-X-ray slopes. The radio-bright objects cluster near the upper left of the diagram; the radio-quiet objects cluster near the lower right of the diagram.

be seen from a comparison of α_{00} and α_{0x} . A correlation coefficient of -0.41 (significant at the 3.1σ level) was found, implying that higher radio emission leads to higher X-ray emission when both are normalized to the optical output (see Fig. 3).

Differences in the X-ray behaviour of radio quasars and optical quasars can be seen more clearly in a plot of the number of sources versus α_{0x} . Figure 4 presents histograms for the 87 detected quasars segregated into: *a*, nearby radio quasars ($z > 1$; $L_r > 10^{41} \text{ erg s}^{-1}$); *b*, distant radio quasars; *c*, nearby optical quasars ($L_r < 10^{41} \text{ erg s}^{-1}$, or undetected); and *d*, distant optical quasars. The total number of objects sampled in each α_{0x} interval, including upper limits, are plotted as dotted histograms in the same figures. The total detected fractions along with $\langle \alpha_{0x} \rangle$, the mean values of α_{0x} , and the standard errors of these means for the detected quasars in each of the subsamples are indicated in the figures. Clearly when the optical quasars are detected in X rays, they are detected with larger values of α_{0x} . For a more quantitative analysis, a simple comparison of the measured means and their standard errors is inadequate because it does not take the upper-limit information into account. A maximum likelihood (ML) technique has therefore been applied to calculate $\langle \alpha_{0x} \rangle$ for the total sample and for each of the subsamples.

We assume that the spectral index α_{0x} is a random variate with distribution function $f(\alpha)$ which is postulated to be independent of other quasar characteristics. Best-fit parameters are determined from the principle of maximum likelihood using standard techniques for treating censored samples and truncated distributions (see, for example, refs 13, 14). Specifically, the likelihood function to be maximized in our case is

$$L = \prod_i f(\alpha_i) \prod_j F(\beta_j) \prod_k f(\alpha_k) / F(\gamma_k) \quad (4)$$

where $F(\beta) = \int_{\beta}^{\infty} f(\alpha) d\alpha$. The first two factors take account of the optically selected quasars, both X-ray detected (*i*) and nondetected (*j*), and the third factor takes account of the quasars (*k*) first discovered as X-ray sources. The quantity β_j is the threshold on the index α_{0x} of the *j*th quasar below which it would have been detected as an X-ray source. Similarly, γ_k is the

threshold on the index of the k th quasar below which quasar-confirming spectroscopic observations would not yet have been made. Where the optical thresholds are known (see, for example, ref. 11), we have used them. Otherwise we have adopted an arbitrary threshold one magnitude fainter than the measured quasar magnitude. For the actual data samples, changing these thresholds by $\pm 1/2$ magnitude changes $\langle\alpha_{ox}\rangle$ by <0.01 .

A nonparametric approach following Avni *et al.*¹⁴ may be used to estimate $f(\alpha)$. However, strict adherence to such a procedure results in $f(\alpha)$ being identically zero outside the range of the measured values for α . If, as is most likely, the present quasar samples do not span the entire range of α s, this unphysical cutoff will introduce a slight bias in the calculated values of α_{eff} . To avoid this source of bias, we take $f(\alpha)$ to be the normal distribution, consistent with the distribution measured for all detected quasars. The sample mean $\langle\alpha_{ox}\rangle$ and the variance σ^2 are then obtained by maximizing L numerically, and the standard errors of these parameters are determined in the usual way from the co-variance matrix. Monte-Carlo simulations have shown that these ML estimators are unbiased.

Using the above procedure, we find for the total sample of 171 quasars a value $\langle\alpha_{ox}\rangle = 1.46 \pm 0.02$. The 26 X-ray-selected quasars with $\langle\alpha_{ox}\rangle = 1.41 \pm 0.03$ are indistinguishable from the remainder of the sample; they also do not differ significantly from other nearby optically selected, radio-quiet quasars (see Fig. 4c). Dividing the sample into radio quasars and optical quasars, we find values of 1.38 ± 0.03 and 1.52 ± 0.03 , respectively. Dividing the two classes into low- and high-redshift groups renders the distinction even more striking. Although there is no difference between the low- and high-redshift radio quasars ($\langle\alpha_{ox}\rangle = 1.36 \pm 0.04$ and 1.40 ± 0.03 , respectively), the optical quasars do show evidence of evolution with $\langle\alpha_{ox}\rangle = 1.44 \pm 0.03$ and 1.65 ± 0.04 , respectively.

Most of the difference in the values of $\langle\alpha_{ox}\rangle$ for the two classes arises from a difference in X-ray luminosity. The luminosity distribution histograms shown in Fig. 5 suggest that, in general, optical quasars have lower X-ray luminosities than radio quasars. The mean log X-ray luminosities are 44.61 ± 0.16 and 45.64 ± 0.13 , respectively, for detected quasars.

We note that a subset of the radio quasars, the flat-spectrum radio sources in general and the OVV's in particular, plays a large part in establishing the distinction between radio quasars and optical quasars. The OVV's are shown as crosshatched objects in Fig. 4. For the 11 OVV's, $\langle\alpha_{ox}\rangle = 1.25 \pm 0.05$, compared with 1.41 ± 0.04 for radio quasars without the OVV's. Discounting the OVV's, the mere absence or presence of radio emission does not strongly correlate with the level of X-ray emission from nearby objects. However, the separation at high redshifts seen in Fig. 4 remains significant.

Discussion

The present data suggest that an instructive segregation of quasars into two types can be made: those with $L_r < 10^{41}$ erg s⁻¹ (Type I) and those with $L_r > 10^{41}$ erg s⁻¹ (Type II). Type I quasars make up $\sim 90\%$ of the total population. For these objects, the typical luminosity in the 3,000–6,000-Å band

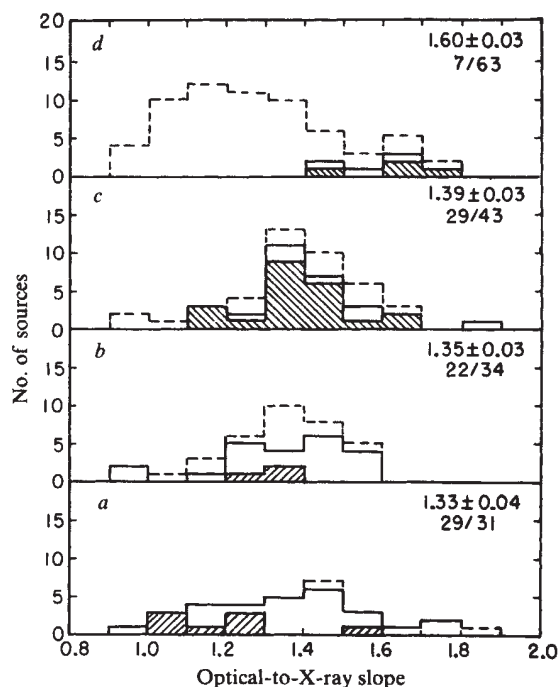


Fig. 4 Optical-to-X-ray slope distributions for: *a*, nearby ($z < 1$) radio-bright ($L_r > 10^{41}$ erg s⁻¹); *b*, distant radio-bright; *c*, nearby radio-quiet or undetected; and *d* distant radio-quiet or undetected quasars. The dashed histograms represent the total number of objects with a detection or a 3σ upper limit in the appropriate α_{ox} bin. The solid histogram represents only those objects which were detected in the X ray. The hatched histograms in *a* and *b* represent the OVV's in our sample. The hatched histograms in *c* and *d* represent X-ray-discovered quasars. The total detected fraction and the mean and standard deviations for the detected quasars in each subsample are given.

ranges from 10^{43} to 10^{47} erg s⁻¹. In the 0.5–4.5-keV soft X-ray regime, their output ranges from 10^{43} to 10^{46} erg s⁻¹ with $\langle\alpha_{ox}\rangle \sim 1.5$ and $\alpha_{r0} < 0.2$. In contrast, Type II quasars are much more luminous, most notably in the radio and X-ray regimes, with $\langle\alpha_{ox}\rangle \sim 1.35$ and $\alpha_{r0} > 0.2$. (If one considers the total energy radiated by several typical Type I and Type II quasars, it is clear that, though substantial overlap exists from one class to the other, Type I quasars are typically 1–2 orders of magnitude less luminous than Type II objects in the range 10^8 – 10^{18} Hz.) Our results, along with previous studies by Schmidt¹⁰ and most recently by Turner¹⁵, indicate that these two types of quasars evolve quite differently: the radio-bright, flat-spectrum type II quasars are consistent with no evolution, whereas radio-quiet Type I objects exhibit a strong luminosity and/or density enhancement at earlier epochs. The parameters of the two classes are summarized in Table 2.

Data from our larger sample of active galactic nuclei provide an important clue to the underlying cause of this class distinction. N-galaxies and BL Lac objects, which are generally bright radio sources, are found to be luminous soft X-ray emitters; we find values of $\langle\alpha_{ox}\rangle = 1.30 \pm 0.07$ and 1.31 ± 0.05 for 15 and 16 observed sources, respectively. These values are consistent with α_{ox} calculated for the few previously discovered BL Lacs¹⁶ and N-galaxies¹⁷. A large number of these active nuclei are associated with underlying elliptical galaxies¹⁸. On the other hand, Seyfert galaxies, which are typically weak radio sources, are also found to have lower X-ray luminosities^{19,20} and a larger $\langle\alpha_{ox}\rangle$ when compared with nearby radio-bright active galaxies. More than 90% of Seyferts are identified with spiral or barred spiral galaxies²¹. This distinction in the morphology of the galaxies underlying Type I and Type II objects may signal differences in the nature of the compact nuclear source and/or its immediate environment.

Table 2 Properties of the two quasar classes

	Type I	Type II
% Of total population	90%	10%
L_r (3.1–8.1 GHz) (erg s ⁻¹)	$<10^{41}$	$>10^{41}$
L_0 (3,000–6,000 Å) (erg s ⁻¹)	10^{43} – 10^{47}	10^{43} – 10^{47}
L_x (0.5–4.5 keV) (erg s ⁻¹)	10^{43} – 10^{46}	10^{44} – 10^{47}
$\langle\alpha_{ox}\rangle$	1.5	1.35
α_{r0}	<0.2	>0.2
Evolution	Strong	Weak
Related AGNs	Seyferts	BL Lacs, N galaxies
Related Hubble type	Spirals	Ellipticals
Time variability	...	$\times 10$ (OVV's)

Our observation that the X-ray flux correlates with other quasar continuum measurements from the UV region to the millimetre region, along with the observation that the mean value of the IR-optical slope of 1.3–1.4 (ref. 22) is similar to $\langle\alpha_{\text{ox}}\rangle$, supports the view that synchrotron radiation from a single power-law distribution of electrons is responsible for most of the 10^{13} – 10^{18} -Hz emission in quasars. The lack of correlation between the emission at X-ray and radio frequencies may be partly due to low-frequency radio emission generally coming from a halo or extended lobe components and thus not measuring the power of the central nuclear source which is presumably responsible for all of the X-ray emission. Furthermore, the compact component may become opaque at frequencies below a few gigahertz due either to synchrotron self-absorption or to free-free absorption by intervening clouds. Absorption by such clouds can also decrease the level of measured soft X-ray emission, although very large column densities ($n_{\text{H}} \geq 10^{23} \text{ cm}^{-2}$ at $z = 3$) would be required to explain the low X-ray outputs of the high-redshift Type I objects.

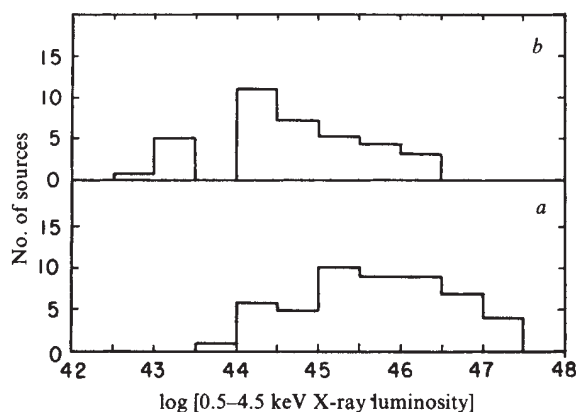


Fig. 5 Histograms of the number of sources plotted against log [0.5–4.5-keV X-ray luminosity (erg s^{-1})] for a, radio-bright objects and b, radio-quiet objects.

Although at soft X-ray energies direct synchrotron radiation seems to be dominant for nearly all quasars, a second mechanism could explain the enhanced X-ray emission from Type II objects in general and OVV in particular. Compton scattering of flat-spectrum centrimetric radio photons off relativistic electrons with a Lorenz factor of $\sim 10^3$ can add significantly to the total X-ray emission above a few kiloelectron volts²³. For the OVVs, the problem has been that the incoherent synchrotron self-Compton (SSC) process predicts high X-ray fluxes that are inconsistent with the upper limits derived from the early 2–10-keV all-sky surveys²⁴. However, the level of X-ray emission we now observe from these sources can be made consistent with the SSC model if the emitting plasma is moving at relativistic velocities with an energy density in the electrons

comparable to that in the magnetic fields⁴. For the non-OVVs, the observation that there is little difference in the X-ray behaviour between the two classes for nearby quasars suggests that the Compton contribution is relatively unimportant below a few kiloelectron volts. Above a few kiloelectron volts, however, a change in spectral slope from ~ 1.5 to a value ≤ 0.5 is expected as the dominant emission mechanism changes from synchrotron to SSC. Recent observations of the X-ray spectra of BL Lac objects^{25,26} support the idea of spectral flattening in the 1–10-keV region. In this picture, the effective power-law index in the spectral band sampled by the IPC is ~ 1 for Type II objects. As there is no K correction for a power law of index one, the lack of any distinction between $\langle\alpha_{\text{ox}}\rangle$ at low and high redshifts for Type II objects follows directly. For Type I objects, only the synchrotron power law with $\alpha_{\text{ox}} \sim 1.5$ is present, explaining the greater difficulty we have in detecting high-redshift Type I objects.

One important consequence of the separation of quasars into two distinct classes is that preliminary conclusions reached by Tananbaum *et al.*⁵ concerning the contribution of quasars to the diffuse X-ray background need to be modified. Because neither the samples of quasars studied by Tananbaum *et al.*⁵ nor by us are statistically complete, we cannot derive an X-ray luminosity function directly but must rely instead on optical quasar counts and $L_{\text{x}}/L_{\text{o}}$ values for different quasars. As the surface density of quasars at faint magnitudes is generally derived from objective prism surveys sensitive to $z > 1.7$ and as most of these sources are not strong radio sources, the proper value of α_{ox} to apply to the optical quasar count is that associated with distant optical quasars. The effective α_{ox} , α_{eff} (see ref. 5), inferred from the mean $L_{\text{x}}/L_{\text{o}}$ ratio for all distant optical quasars, is 1.63 ± 0.04 . This value means that, on average, optical quasars contribute a factor of 8 less to the diffuse X-ray background than the value of $\alpha_{\text{eff}} = 1.27$ used by Tananbaum *et al.*⁵. If Type II objects comprise 10% of all quasars, their value of $\alpha_{\text{eff}} = 1.26 \pm 0.03$ implies that they contribute $\sim 70\%$ of the amount from all optical quasars. Treating the OVVs separately may alter the estimate, but the net result is to reduce the contribution from all quasars by a factor of ≥ 4 from the value given by Tananbaum *et al.*⁵. To explain the entire diffuse soft X-ray background as resulting from quasars, then, one must integrate the optical $\log N$ – $\log S$ relation to $m_{\text{B}} = 21.5$ and assume that the slope of the $\log N$ – $\log S$ curve remains steeper than 2. Similar results have been reported by Zamorani *et al.*²⁷.

We believe that the data presented here support a picture incorporating the differing importance of synchro-Compton radiation in Type I and Type II quasars, possibly enhanced to some degree by an excess of absorbing material in the radio-quiet objects. While simultaneous spectral and time variability studies over as wide a frequency range as possible are essential for further progress in this field, it is clear that X-ray observations will play a major part in our future understanding of the evolution of quasars, their connection to other active galaxies, and their precise contribution to the diffuse X-ray background.

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Cordilleran suspect terranes

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Over 70% of the North American Cordillera is made up of 'suspect terranes'. Many of these geological provinces are certainly allochthonous to the North American continent and seem to have been swept from far reaches of the Pacific Ocean before collision and accretion into the Cordilleran margin mostly in Mesozoic to early Cenozoic time.

RECENT tectonic studies and a new geological and geophysical data base support earlier theories¹⁻³ that much of the North American Cordillera is a vast mosaic or collage^{4,5} of what are here termed 'suspect terranes'⁶. The terranes are suspect because we cannot be certain of their palaeogeographical setting with respect to North America through much of Phanerozoic time. Most are truly allochthonous and seem to have collided and accreted to the North American cratonic margin mostly during Mesozoic and early Cenozoic time⁷. Large-scale concurrent and post-accretionary horizontal translations of hundreds of kilometres⁸ and significant rotations around vertical axes^{9,10} are also indicated, both of which are continuing to the present.

Distribution and character of terranes

The distribution of the major Cordilleran suspect terranes is shown in Fig. 1. More than 50 terranes are recognized some of which are here grouped for simplicity and designated composite terranes. Some terranes are too small to show at the scale of this map.

Once the Palaeozoic miogeocline is identified in the geology of western North America all geological terranes outside it are inherently suspect in that their palaeogeographical distribution or their palaeotectonic setting with respect to North America is uncertain. The reason is based on a simple plate tectonic concept. During most of latest Precambrian through early Palaeozoic time the western margin of North America was a passive continental margin across which was draped a growing miogeoclinal terrace¹¹ as some proto-Pacific Ocean opened. Brief periods of convergence and collision are inferred during mid-Palaeozoic time¹², but the progressive out-building of the terrace was almost uninterrupted for at least 700 Myr. This regimen changed in latest Triassic to middle Jurassic time¹³ and since then the margins of the Pacific Ocean have been convergent or transform. Subduction related to convergence consumed all of the vast Palaeozoic Pacific Ocean¹² and it follows that all Palaeozoic terranes now found outside the edge of the original Palaeozoic passive continental margin must have somehow accreted against that margin during Mesozoic-Cenozoic time. It also follows that all younger geological terranes outside that margin (many of which sit on older Palaeozoic rocks) are also suspect, until proved otherwise, and may have been accreted against that margin.

The terranes shown in Fig. 1 are characterized by internal homogeneity and continuity of stratigraphy, tectonic style and history. The boundaries between terranes are fundamental discontinuities in stratigraphy that cannot be explained easily by conventional facies changes or unconformity. Most boundaries separate totally distinct temporal or physical rock

sequences^{15,19}, and many juxtapose different faunas¹⁶. Some terranes carry palaeomagnetic records that differ strongly from those of cratonic North America^{9,10}. These data suggest large displacements and/or rotations between the terranes themselves and between the terranes and stable North America.

Note that identification of a terrane is based primarily on its stratigraphy¹⁴ and need not carry any genetic or even plate tectonic implication. At the start of investigations, the identified terranes are simply considered as domains in the descriptive sense. Their identification and description has provided great insight into Cordilleran geological and tectonic history.

In support of the concept of terranes and their suspect character it is important that most of them display sedimentary and volcanic rock sequences that are of oceanic affinity rather than continental. It is also significant that rocks proved to be older than middle Palaeozoic are not common in the larger suspect terranes¹⁵. Most contain only upper Palaeozoic and/or Mesozoic sequences and most seem to have formed far removed from any continental influence, or at best at the most distal reaches of continental influence.

Most of the boundaries between terranes are known or suspected faults that usually display complex structural history (for example, see ref. 19). Many of the boundaries are certainly sutures, now largely cryptic, and most have been reactivated by concurrent and post-collisional large-scale, right-lateral strike-slip movements. High-pressure mineral assemblages of the blueschist facies from some of these boundaries have yielded Mesozoic radiometric ages⁵. Also, several boundaries are marked by thick and highly deformed Mesozoic sequences of turbidite flysch⁷.

The allochthonous terranes are of several types. A few are, or contain, pieces of oceanic crust (Ch, Cl, Br, SJ, BL, Trp, C) and represent last vestiges of large late Palaeozoic-early Mesozoic oceans now trapped well within the Cordillera far from the present Pacific margin¹⁵. The Permian Tethyan faunas characteristic mainly of the Cache Creek terrane are totally distinct from coeval faunas found on both sides¹⁶. Other terranes contain fragments of oceanic arcs subsequently swept against the Cordilleran margin (R, I, G, YT, W, P, St, E, Ca, BL, KL, S, Si). A significant proportion of these arcs are late Palaeozoic in age, many are late Triassic to Jurassic in age, but at least one (the Gravena-Nutzotin belt)¹⁷ is as young as early Cretaceous. The major Palaeozoic and Mesozoic arcs (St, P, W) have no known basement other than presumed oceanic crust. Some of the arcs, although submarine in character, rode passenger on older basement terranes. Other terranes seem to be fragments or slices off distal parts of unknown continental edges (E, NF, PM, YT, Ax, Sa, RM) and they possess what might be termed a

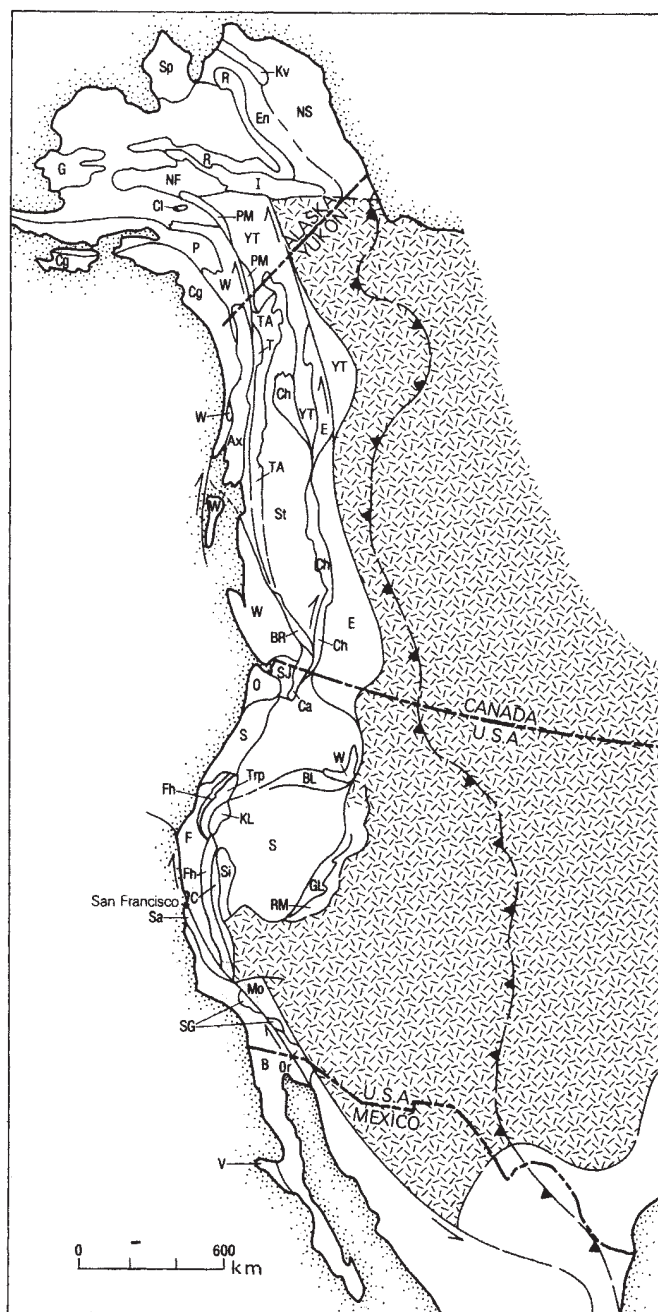


Fig. 1 Generalized map of Cordilleran Suspect Terranes. Dashed pattern, North American autochthonous cratonic basement. Barbed line, eastern limit of Cordilleran Mesozoic-Cenozoic deformation. Barbed arrows, direction of major strike-slip movements. Terranes are described below.

Alaska

(for further information on the distribution and character of terranes in Alaska, see refs 14, 17–20, 53)

- Sp, Seward Peninsula—structurally complex assemblage of Precambrian metamorphic and sedimentary rocks, and Palaeozoic carbonate rocks.
- Ns, North Slope—Precambrian, Palaeozoic, and Mesozoic clastic and carbonate sequence—part of North America, but may have moved from original position.
- Kv, Kagvik—Thin sequence of radiolarian chert, argillite, shale, and minor volcanics, Mississippian to Triassic in age.
- En, Endicott—metamorphosed Lower to Upper Palaeozoic clastic and carbonate rocks intruded by Palaeozoic granitic rocks.
- R, Ruby—composite terrane comprising at least three separate units, including Precambrian metamorphic rocks, mid to Upper Palaeozoic volcanic and sedimentary rocks, and thick piles of Lower Mesozoic basalt and chert.
- I, Innoko—structurally deformed sequence of Upper Palaeozoic to early Mesozoic chert, argillite, graywacke, and basic to intermediate volcanics.
- NF, Nixon Fork—Precambrian metamorphic rocks overlain by Palaeozoic and Mesozoic carbonate, clastic, and cherty rocks.

- G, Goodness (composite)—includes three terranes: (1) a complex assemblage of deformed Upper Palaeozoic volcanics, chert, and graywacke with blocks of older limestone; (2) Precambrian gneisses and schist; and (3) Mesozoic arc-derived volcanic flows, tuff, and graywacke, with interbedded chert.
- CI, Chulitna (composite)—includes three terranes: (1) Devonian ophiolite overlain by Palaeozoic chert, volcanic conglomerate, limestone, and flysch, and Mesozoic limestone, redbeds, flysch, and chert; (2) Mesozoic chert, argillite, crystal tuff, and conglomeratic sandstone; (3) Upper Palaeozoic tuff, and chert, volcanic graywacke, with blocks of Lower Palaeozoic limestone.
- PM, Pington & McKinley (composite)—includes three terranes: (1) Upper Palaeozoic phyllite and Triassic thin-bedded limestone and sooty black shale; (2) Upper Palaeozoic chert, Triassic pillow basalt, and Upper Mesozoic flysch and conglomerate; (3) Lower Palaeozoic limestone; tuff and flysch of unknown ages.
- YT, Yukon-Tanana (composite)—includes regionally metamorphosed schist and gneiss of Precambrian(?) age, Devonian limestone, Upper Palaeozoic silicic metavolcanic rocks, Permian ophiolite, and foliated granitic rocks of unknown age.
- W, Wrangellia—Upper Palaeozoic arc complex composed of flows, breccias, and volcanoclastic rocks overlain by limestone, clastics, and chert, and Mesozoic pillowed and subaerial basalt flows succeeded by limestone, cherty limestone, and clastic rocks.
- P, Peninsular—rare Palaeozoic limestone, Triassic basalt, argillite, and limestone, Lower Jurassic volcanic and volcanoclastic rocks, younger clastics.
- Cg, Chugach (composite)—includes (1) deformed Upper Mesozoic flysch and melange units, and (2) deformed Lower Cenozoic flysch and volcanic rocks.
- Ax, Alexander—complex terrane of Precambrian(?) and Palaeozoic volcanic rocks, clastics, and limestone, and Mesozoic volcanics, limestone, and clastic rocks.
- T, Taku—structurally complex assemblage of Upper Palaeozoic volcanoclastics, limestone, flysch(?), and Lower Mesozoic basalt, limestone, and flysch.
- TA, Tracy Arm—structurally complex assemblage of marble, pelitic gneisses, and schist of unknown ages.

Canada

(for further information on distribution and character of terranes in Canada, see refs 2, 5, 8, 15, 16, 19, 25, 33)

- Ch, Cache Creek terrane—Mississippian to Middle (Upper?) Triassic, highly disrupted radiolarian chert, argillite, basalt, alpine-type ultramafics, large shallow-water carbonates, and local blueschist metamorphism.
- BR, Bridge River terrane—Middle Triassic to Lower Middle Jurassic, highly disrupted radiolarian chert, argillite, basalt, alpine-type ultramafics and minor carbonate.
- St, Stikine terrane—Mississippian and Permian volcanoclastics, basic to acidic volcanics and carbonates, locally deformed and intruded in middle to late Triassic time, overlain by Upper Triassic to Middle Jurassic volcanogenic strata.
- E, Eastern assemblage (composite)—includes possible late Precambrian-early Palaeozoic metamorphic terranes, of possible continental affinity, together with Mississippian to Triassic basalt, ultramafics and chert and volcanoclastics and carbonates, overlain unconformably by Middle Triassic to Lower Jurassic volcanogenic strata.

Washington and Oregon

- SJ, San Juan (composite)—includes highly deformed Mesozoic chert, argillite, graywacke, and volcanic rocks, partly in melanges, with blocks of lower Palaeozoic plutonic rocks, Palaeozoic chert, carbonates, and volcanic rocks. Permian limestone blocks contain Tethyan fusulinids (see ref. 54).
- Ca, Northern Cascades (composite)—includes crystalline and pelitic gneisses, and thrust sheets composed of (1) Upper Palaeozoic andesitic volcanics and associated sedimentary rocks; (2) green schist and blue schist; and (3) Jurassic ophiolite (see ref. 55).
- O, Olympic—Lower Cenozoic volcanic rocks and associated deep and shallow water sedimentary rocks. Basement unknown, but presumed to be oceanic (see ref. 56).
- S, Lower Cenozoic volcanic and sedimentary rocks lying west of the Cascade Range. Palaeomagnetic data imply post-Eocene clockwise rotation of 70° (see refs 35, 36).
- BL, Blue Mountains (composite)—includes melange with blocks of Palaeozoic ophiolite, limestone, and chert, and Mesozoic chert and sandstone, structurally overlain by Triassic and Jurassic volcanic sandstone, conglomerate, and argillite (see refs 57, 58).

California

- Fh, Foothills—Upper Jurassic andesitic volcanic and volcanoclastic rocks associated with phyllite, slate, and graywacke, and Upper Jurassic ophiolite (see refs 59, 60).
- Trp, Triassic and Palaeozoic of Klamath Mountains (composite)—includes a structurally complex assemblage of Lower Mesozoic ophiolite, chert, basalt, Jurassic andesitic rocks, and associated sedimentary rocks (see refs 61, 62).
- KL, Eastern Klamath Mountains—Middle to Upper Palaeozoic clastic, volcanic, and carbonate rocks, overlain by Triassic and Jurassic volcanics and minor limestone (see refs 63, 64).

- Si, Northern Sierra—Lower Palaeozoic clastic sedimentary rocks, Upper Palaeozoic and Lower Mesozoic volcanic and associated sedimentary rocks (see ref. 65).
- C, Calaveras (composite)—including a western belt of melange with ophiolite and Mesozoic chert, and an eastern belt of quartzose clastic rocks, argillite, and minor Permian limestone (see ref. 66).
- F, Franciscan (composite)—includes Upper Mesozoic Great Valley sequence with ophiolite at base, and structurally underlying disrupted and partially metamorphosed rocks of the Franciscan Complex (see ref. 67).
- Sa, Salinia—includes metamorphosed pelitic rocks, marble, and graywacke of unknown ages, intruded by Cretaceous granite plutons (see ref. 68).
- SG, San Gabriel (composite)—two structurally complex and juxtaposed Precambrian crystalline terranes intruded by Mesozoic plutons (see ref. 69).
- OR, Orocopia—metagraywacke and mudstone and minor chert and basic volcanic rocks, age unknown. No known basement (see ref. 70).
- Mo, Mohave (composite)—juxtaposed and disrupted Palaeozoic sedimentary sequences, Lower Mesozoic sedimentary and volcanic rocks intruded by Mesozoic plutons (see ref. 71).

Mexico

- B, Baja—includes scattered localities of Upper Palaeozoic limestone and Lower Mesozoic clastic rocks, overlain by a thick pile of Upper Mesozoic volcanic and volcanoclastic rocks, capped by latest Cretaceous quartzofeldspathic sandstone (see ref. 72).
- V, Vizcaino (composite)—includes Triassic basalt, chert, and limestone, Upper Jurassic arc-derived volcanic and volcanoclastic rocks, Upper Jurassic and Cretaceous clastic rocks, ophiolite, and structurally underlying Upper Mesozoic blue schist and disrupted rocks similar to the Franciscan Complex (see ref. 73).

Nevada

- S, Sonoma (composite)—includes Upper Palaeozoic volcanics in the south, and Lower Mesozoic volcanics in the north. Si and KL terranes originally included in Sonoma (see ref. 74).
- GL, Golconda—structurally deformed assemblage of chert, argillite, minor limestone, and volcanics of Mississippian to Permian age (see ref. 75).
- RM, Roberts Mountains—structurally complex assemblage of chert, argillite, sandstone, basalt, and minor limestone of Cambrian to latest Devonian or early Mississippian ages (see ref. 76).

'quasi-continental' character. A few terranes are of uncertain origin. A significant number of the terranes have basalt or gabbroic rocks, particularly of late Triassic age (I, PM, W and so on) suggesting they are fragments from rifting events, intraplate volcanism, or oceanic plateaus of unknown origin or setting¹⁸.

In a few cases it can be shown by stratigraphical and structural controls that disparate terranes amalgamated before final accretion against the North American margin^{18,19}. One of the largest and best documented cases is the amalgamation of Wrangellia²⁰ with the Alexander terrane. Wrangellia everywhere comprises a late Palaeozoic submarine arc assemblage, with no known basement, overlain by a distinctive and very thick (up to 6,000 m) partly submarine, partly subaerial Upper Triassic basalt, overlain in turn by sedimentary rocks which extend into the early Jurassic. Palaeomagnetic data from identical Upper Triassic basalt sequences in eastern Oregon (J. Hillhouse, personal communication), Vancouver Island in western Canada²¹, and from southern Alaska²² all record the same low Triassic palaeolatitudes with respect to Triassic North America. The Alexander terrane is a complex assemblage of volcanosedimentary rocks of Palaeozoic age¹⁷. Somewhere on route from its equatorial origins, Wrangellia amalgamated with the Alexander terrane^{3,19}, as demonstrated by the overlapping Upper Jurassic and Cretaceous strata of the Gravina-Nutzotin belt, and with the Lower Jurassic arc of the Peninsular terrane¹⁸. Once amalgamated the resulting super-terrane was the site of variably distributed Jurassic to Lower Cretaceous Gravina-Nutzotin¹⁷ arc activity before its final consolidation into the Cordilleran orogen. Since accretion Wrangellia has been fragmented by major horizontal translations and rotations so that its pieces are now scattered over almost 2,000 km of the Cordilleran margin from Oregon to Alaska²⁰.

Some terranes are very small (most are not shown on Fig. 1) and have no known counterparts in the Cordillera. A remarkable example is in the Chulitna terrane²³ in southern Alaska. It lies with other small, but totally distinct, terranes embedded in a mass of late Jurassic to early Cretaceous flysch north of the Wrangellia terrane in the Central Alaska Range. Only several

tens of kilometres long, the terrane is a nappe-like structure exposing in continuous sequence late Devonian ophiolite, Mississippian chert, Permian volcanic conglomerate and breccia, flysch, chert and limestone, Lower Triassic limestone, Upper Triassic red beds and basaltic to silicic volcanics, Jurassic sandstone and chert, and Cretaceous argillite, chert, sandstone and coquinoid limestone. No other stratigraphic section such as this is known anywhere in the Cordillera. Lower Triassic ammonites from this terrane are of equatorial affinity²⁴ and indicate a southern origin.

Not shown in Fig. 1 are some overlap assemblages termed superjacent terranes. These terranes are sedimentary and volcanic sequences deposited on the basement terranes shown in Fig. 1 and tie together previously disjunct terranes. The Gravina-Nutzotin belt¹⁷ is an example. Analyses utilizing basement and superjacent stratigraphy, biostratigraphy and palaeomagnetic data combined with consideration of belts of dated cross-cutting plutonic rocks provide critical data for unraveling the pre-, syn-, and post-accretionary history of these complex structural units.

Accretionary history

A full understanding of the history of accretion and post-accretionary consolidation of Cordilleran suspect terranes into the North American Cordillera is incomplete. Although we review here what is known or suspected for the assembly as a whole, much of what we say is preliminary as additional complexities continue to unfold.

Southern Alaska is made up of several large terranes with numerous smaller ones scattered along the margins and between the larger ones^{18,23}. The Yukon-Tanana terrane is largely made up of heterogeneous gneiss and schist, scattered deformed plutons, and high-level sheets of chert, basalt and ultramafic rock. The terrane is probably composite with nappes of upper Palaeozoic oceanic assemblages thrust across a quartz-feldspathic and silicic volcanic-rich protolith of probable Precambrian to known Palaeozoic age and of unknown continental affinity. The time of final emplacement of the composite terrane against North America is not known, but may have been as recent as Cretaceous time with earlier amalgamation in the Triassic²⁵. Subsequently, in post-Early Cretaceous time Wrangellia, already amalgamated with the Lower Jurassic Peninsular arc terrane and Alexander terrane, collided with the Yukon-Tanana terrane entrapping many smaller terranes of unknown origin (including Chulitna terrane) within a flysch-filled suture zone^{18,26}. The successive accretions must have caused subduction systems to step southward and from late Cretaceous to early Tertiary time, the Chugach and younger accretionary flysch prisms were emplaced against the newly formed North American margin²⁷. Concurrent and post-accretionary convergence has caused translation and intra-plate deformation with reactivation of old sutures. This has resulted in northward strike-slip displacements of hundreds of kilometres along the Tintina, Denali and other faults in Alaska and adjacent Canada^{25,28-30}.

The largest allochthonous terrane in Canada is the Stikine terrane¹⁵. It has a basement of upper Palaeozoic submarine arc rocks overlain by Upper Triassic to Middle Jurassic submarine and subaerial volcanic and sedimentary rocks (Takla-Hazleton assemblage)¹⁵ and intruded by coeval granitic plutons. This major block seems to have accreted between early Triassic and mid-Jurassic time^{25,31} entrapping between itself and the North American margin Tethyan ocean floor (Ch) and a collage of Palaeozoic 'oceanic' arcs and possible distal fragments of the Cordilleran continental terrace (E). The late Jurassic and Cretaceous Bowser Basin³² overlap assemblage is deposited on Stikine terrane and records the first clear sediment source to the east, of both continental and oceanic (Ch) character. Final stages of the accretionary process were emplacements of nappes eastwards and westwards from the suture zone and on to the former continental margin. This eastern accretion was followed by the arrival of Wrangellia⁷, which, from Vancouver Island north,

seems to have been in middle Cretaceous time. In eastern Oregon, its arrival time may be earlier. The northern part of Wrangellia had already amalgamated with the Alexander terrane, as explained above, and arrived bearing a Jurassic arc and the Gravena-Nutzotin late Jurassic to early Cretaceous submarine arc terrane on it. Subsequent thrusting and northward translation on intra-plate strike-slip faults^{8,33} largely during late Cretaceous to early Tertiary time, have disrupted the original relationships, but left a detached fragment of Wrangellia isolated in eastern Oregon. Once again, subduction zones must have stepped outboard through successive accretions, for by late Cretaceous–early Tertiary time the trench was on or near the present Pacific margin⁸.

Much of the central western part of the Cordillera in the US is underlain by several terranes (S, BL, KL, Si, Trp, C) whose very poorly exposed relationships to one another are still imperfectly understood³⁴. Certainly the Permian Tethyan fauna-bearing terranes of Oregon and California are important and which move to positions much closer to the Pacific margin as they are tracked southward from their more internal position in Canada. No Tethyan fauna-bearing terranes are known south of central California. The Roberts Mountain allochthon of west-central Nevada is probably a distal continental terrace or marginal oceanic assemblage emplaced across the Cordilleran miogeocline in middle Palaeozoic time¹². This is the oldest known example of accretion in western North America. Most of the remaining suspect terranes in the northern Sierra Nevada, Klamath Mountains and western Nevada are Palaeozoic to Mesozoic volcanic and sedimentary sequences of oceanic and arc-trench affinity which were swept against the Cordilleran margin in late Palaeozoic to middle Mesozoic time⁷. By mid-Cretaceous time the Franciscan accretionary assemblage was forming on the outboard margin of North America. Siletzia, a submarine basalt province of early Tertiary age in western Oregon certainly rotated clockwise over 70° since Eocene time^{35,36} and was only finally emplaced in mid-Tertiary time.

In southwestern North American Precambrian basement extends nearly to the present coastline in southern California and northwestern Mexico. This precludes major large accretionary masses there, but several smaller displaced terranes (Sa, SG, Or) of both oceanic and quasi-continental character are known along this margin. Another important relationship is that some Jurassic magmatic arc terranes were intruded into, or deposited on, North American cratonic basement in northwestern Mexico and the southwestern US. This is in direct contrast to arcs of similar age north of central California which are ubiquitously found outside confirmed North American basement and intruded into or deposited on the suspect terranes themselves. As stated earlier, this makes the northern Mesozoic arc terranes as suspect as the terranes they sit on until it is proved otherwise. How all these Upper Triassic to Jurassic arcs relate to one another along strike and which way they faced are problems currently under much discussion and investigation. Note that major low-angle thrusting in southern California and southwestern Arizona is emerging from recent work and large-scale emplacement of allochthons in this region is not precluded^{37,38}.

The major displaced terrane in southwestern North America so far proposed is of totally different character from those described northwards in the Cordillera. This terrane is that part of the North American craton which is supposed to have moved up to 800 km southeastwards relative to stable North America along the Mojave–Sonora megashear³⁹ or transform fault. This movement is said to have taken place in late Jurassic time and was kinematically linked to the opening of the Atlantic Ocean and the Gulf of Mexico as Africa–South America separated from North America.

Conclusions

The collisions and accretions in western North America discussed above have profound implications for Cordilleran tecto-

genesis^{25,40}. The mechanical process of these accretions and their effect on the accreted masses themselves and on the Cordilleran margin are poorly understood. Certainly thrust faulting has played a dominant part and if the thrust faulting within the accreted terranes is linked to the thrust belts^{41,42} along the eastern Cordilleran margin as has been suggested²⁵, the resulting telescoping is unprecedented in Cordilleran tectonic thought. Concurrent and post-accretionary telescoping and consolidation apparently took place by major intra-plate thrusting and strike-slip translations²⁵. The entire process seems to have endured over a period of at least 120 Myr or from mid-Jurassic well into early Tertiary time. Much northward translation and clockwise rotation occurred which suggest oblique convergence from a general northward Pacific 'mega-drift'⁷, as implied by palaeomagnetic data.

The analogy with the Himalayan orogen and the internal disruption of Asia⁴³ is striking, but there are major differences. Unlike India, a large continental mass which remains coupled to the Indian Ocean plate as the subcontinent indents Asia, the comparatively smaller accreted terranes in western North America became uncoupled from Pacific Ocean plates by formation of subduction zones near the present Pacific margin in late Mesozoic time. Much of the telescoping and translation continued inside the marginal subduction zones well into Tertiary time. During the late Cretaceous to early Tertiary period of Laramide deformation⁶ possible elevated convergence rates between North America and the Farallon and Kula plates combined with variably dipping subducting slabs⁴⁴ may have produced deep-seated Laramide tectonism and complex shifting magmatic patterns across the Cordilleran foreland as a final stage to consolidation of the Cordilleran mosaic and its cratonic foreland. Also North America's generally northward and westward motion over the mantle against the generally northward-moving accretions may have been dynamically important^{41,42,45}. It is not accidental that Cordilleran telescoping on the foreland only began after the Middle Jurassic initiation of opening of the Central Atlantic Ocean which sent the North American plate northward then westward over the Pacific Ocean^{41,8}. Post-middle Tertiary successive overriding of Pacific spreading centres by North America has produced the complex transform regimen which has affected North America's western edge down to the present⁴⁶.

The numerous upper Palaeozoic and lower to middle Mesozoic arcs that so characterize the allochthonous terranes are of special interest. None of these can be confirmed to have stood on the North American cratonic margin. They may have stood offshore in the manner of the present western Pacific Ocean arcs, but if they did we still do not know which way they faced. In any event they would have to have eventually collapsed against the North American margin by subduction of various sorts of small ocean basins caught behind them. This view is attractive because of the example of the western Pacific Ocean, but it does present some difficulties. The principal difficulties are the scraps of Tethyan ocean floor caught inside these arcs and preliminary palaeomagnetic evidence^{9,10,47,48} which suggest large northwards translations of some of these arcs before final accretion. All this presents the possibility that the arcs may indeed be far travelled and totally unrelated to North America and thus exotic fragments from far reaches of the Pacific Ocean⁷. It is not until Cretaceous time that a semi-continuous magmatic arc along the Cordilleran margin can be substantiated by plutonic, volcanic and stratigraphical data^{6,52}.

Either of these models is consistent with possible plate palaeogeographies of the time. During late Palaeozoic and early Mesozoic time two-thirds of our planet's surface was paved with a single enormous ocean⁴⁹. The other third was Pangea. Assuming that lengths and spacings of spreading centres were similar then to that now, a single large ocean would statistically favour more percentage area of old ocean crust lying around ready to be subducted than at any other time before or since the Phanerozoic. If offshore and/or intra-oceanic arcs correlate with subduction of cold and dense old oceanic crust, as has been

suggested⁵⁰, large parts of the late Palaeozoic–early Mesozoic palaeo-Pacific Ocean could have been festooned with magmatic arcs⁵¹. As Pangea began to break up and North America began to advance over that Ocean, the arcs could have been swept northwards against large sectors of North America's margin to

produce much of the Cordilleran mosaic which has been considered here. During this process, the Pacific was cleared of older arcs, oceanic plateaus and continental fragments, leading thus to the creation of the simple plate configuration that characterizes the present eastern Pacific.

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Voltage-dependent translocation of the asialoglycoprotein receptor across lipid membranes

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A membrane receptor protein for asialoglycoproteins induces voltage-dependent increases in ion conductance across a lipid bilayer, probably reflecting penetration of the protein into the bilayer towards an electrically positive pole. In the presence of a specific ligand for the receptor, this penetration leads to a 'translocation' of the receptor from one side of the bilayer to the other. These observations suggest a mechanism by which biological membranes might regulate the disposition of their proteins, and a way in which membrane receptors involved in endocytosis might be spared lysosomal destruction in order to be recycled to the plasma membrane.

BOTH the lipid and the protein components of biological membranes seem to be asymmetrically distributed¹. A key question is whether the biosynthetic process determines once

and for all the orientation of a given membrane component, or whether later processes can affect the component's disposition. At least some membrane lipids do seem to redistribute between

the inner and outer leaflets of the bilayer in a relatively rapid process whose nature is at present not clear². We show here that a membrane receptor protein for asialoglycoproteins induces voltage-dependent increases in ion conductance across a lipid bilayer, probably reflecting penetration of the protein into the bilayer towards an electrically positive pole. In the presence of specific ligand for the receptor, this penetration leads to a 'translocation' of the receptor from one side of the bilayer to the other. These observations suggest that the membrane potential might have a role in regulation of the disposition and function of membrane proteins.

To investigate the assembly and disposition of proteins in membranes we have studied the interaction with lipid bilayers of a membrane receptor purified from rabbit hepatocytes. This hepatic binding protein (HBP) has been studied extensively³. When serum glycoproteins are desialylated to expose penultimate galactose moieties, they are removed from circulation by binding to this receptor, with subsequent endocytosis.

A general outline of receptor-mediated endocytosis is the following. After the ligand has bound to the receptor it is rapidly endocytosed and delivered to a lysosome, where it is degraded. The process has a half life of 15–60 min. The receptor, on the other hand, has a half life of ~88 h (ref. 4). This raises a topological problem: the receptor's binding sites face outwards from the plasma membrane. After ligand binding and endocytosis, they would be expected to face the inside of the lysosome and to be destroyed. However, evidence⁴ suggests that the receptor in fact faces the outer, cytosolic surface of vesicles in the lysosome-enriched fraction. Thus the receptor may cross the membrane at some point during endocytosis, and we present here a possible mechanism for such a translocation.

Rabbit HBP can be purified to homogeneity and, as shown by SDS polyacrylamide gel electrophoresis, has two subunits, of molecular weights 40,000 and 48,000 (ref. 5). By precipitation with ethanol it can be obtained in water-soluble form, free of detergent and of bilayer lipid. Our previous studies⁶ showed that this protein forms a stable association with phosphatidylcholine vesicles merely by mixing of the protein with vesicles. Here we report the interaction of HBP with black lipid membranes (BLMs). Advantages of the BLM were the accessibility of both sides of the membrane for addition of reagents and the ease with which the membrane potential could be controlled. Partial insertion of a protein into a BLM can perturb the membrane's structure and increase the permeation of ions across it (that is, increase its conductance)⁷.

The nature of HBP-induced BLM conductance

Figure 1 shows an asymmetrical conductance increase seen when HBP was added to one side (*cis*) of a BLM. When the potential was negative on the *trans* side, the conductance was that of an unmodified BLM. When the potential was positive on the *trans* side, HBP induced a conductance increase to about 10 times that of the unmodified BLM. In most such experiments the conductance changed to 10–100-fold the unmodified BLM level (see below). Conductance changed in the form of rapid fluctuations rather than in the discrete, uniform steps characteristic of channels or the fast, smooth rise characteristic of carriers⁸. This form suggests that the conductance was induced by a perturbation of membrane structure⁷ rather than by an ionophore associated with HBP. Figure 1 shows the asymmetrical time dependence and voltage dependence of the conductance increase. In Fig. 1a the voltage was switched at time intervals of about 30 s between +60 and –60 mV. During *trans*-positive intervals the conductance increased, and during *trans*-negative periods it decreased. The time required for conductance increase was much longer than that for the decrease. When the voltage was imposed in the form of a triangular wave (Fig. 1b), the result suggested a threshold effect; that is, the conductance started to rise steeply as the voltage increased and then decreased sharply as the voltage fell below 20–30 mV *trans*-positive. With a *trans*-positive voltage above threshold the conductance increased with time (Fig. 2). If the voltage was held below threshold a low conductance could be maintained for hours.

The diffusion potential of an HBP-modified BLM, measured as a function of potassium nitrate concentration ratio, showed a slope of about 10 mV per decade of concentration ratio $[K^+]_{cis}/[K^+]_{trans}$, indicating a slight selectivity for cations over anions.

The suggestion from the BLM experiment (Fig. 1) that HBP moves towards a *trans*-positive potential was supported by studies of HBP inserted into phospholipid vesicles by methods described elsewhere⁶. We determined the exposure of the protein's tryptophan groups to aqueous quenchers, iodide and acrylamide. The fluorescence intensity of the tryptophan residues in vesicle-associated HBP decreased with increasing concentration of quencher. When we imposed a *trans*-positive voltage gradient across the vesicles by adding the K^+ -selective ionophore valinomycin in the presence of a K^+ gradient, the quenching was markedly reduced, suggesting a voltage-

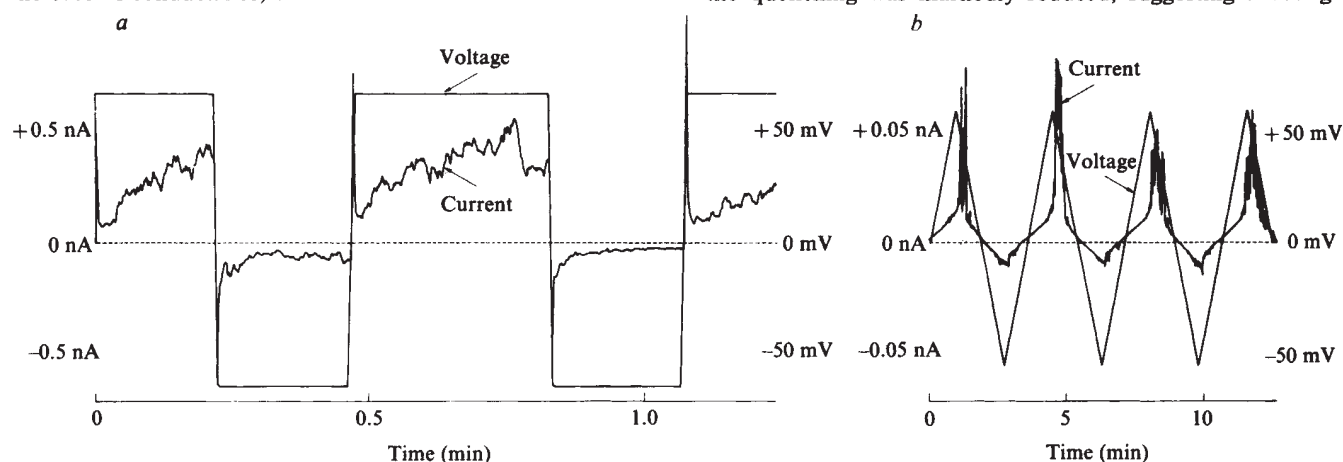


Fig. 1 Asymmetric conductances in a HBP-modified BLM. The BLM was formed from a solution of oxidized cholesterol (2% in *n*-decane) applied across an aperture (1-mm diameter) in a Teflon partition separating two 3-ml chambers, according to the procedure developed by Mueller and Rudin²⁴. Each chamber contained an aqueous solution of KCl (6 mM), NaCl (139 mM), and HEPES (10 mM) at pH 7.4. The solution contained about 50 μ M Ca^{2+} . HBP (final concentration 10 μ g ml⁻¹) was added to one compartment (*cis*) and the solution was stirred by means of Teflon-covered magnets turned by a d.c. motor fixed below the bilayer chamber. The records were taken immediately after adding the HBP. The initial conductance was 0.1 nS. The area of the BLM was 8×10^{-3} cm². The voltage was applied from a d.c. battery switched at arbitrary times by hand (a) or in the form of a triangular wave at a frequency of 0.003 Hz using a function generator (Hewlett-Packard 3310B) (b). Current was passed through the BLM by means of Ag/AgCl electrodes and fed into a current transducer that converted membrane current (*I*) into a voltage signal. The output of the voltage source and of the current transducer were fed into two channels of a d.c. recorder (Leeds and Northrup 624). The voltage is defined as $\psi_{trans} - \psi_{cis}$. Note that in Fig. 2b there is a small capacitive current.

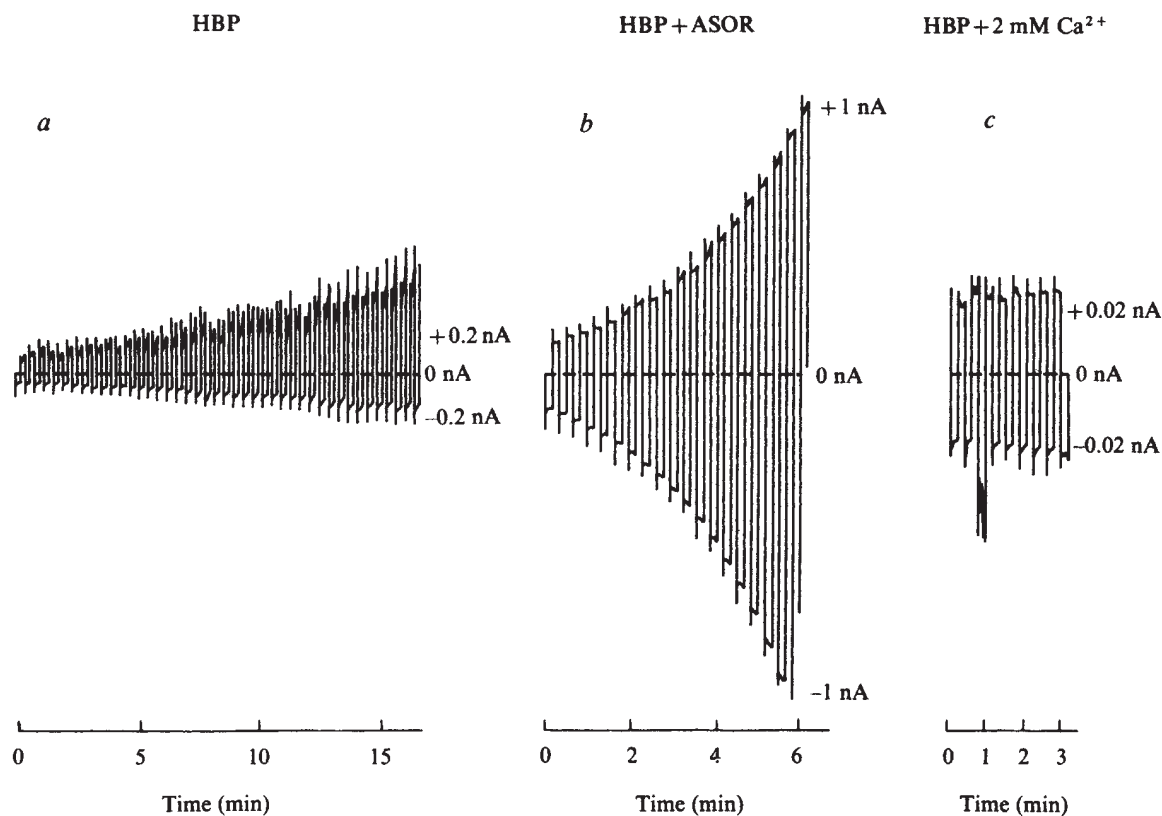


Fig. 2 Symmetrization of conductance on addition of specific ligand or Ca^{2+} to an HBP-modified BLM. The conditions were as described in the legend to Fig. 1. Voltage (not shown) was applied in a rectangular wave form alternating between +50 and -50 mV, at a frequency of 0.04 Hz. *a*, The same buffer as in Fig. 1; *b*, $10 \mu\text{g ml}^{-1}$ ASOR added to the *cis* side; *c*, 2 mM Ca^{2+} present on both sides.

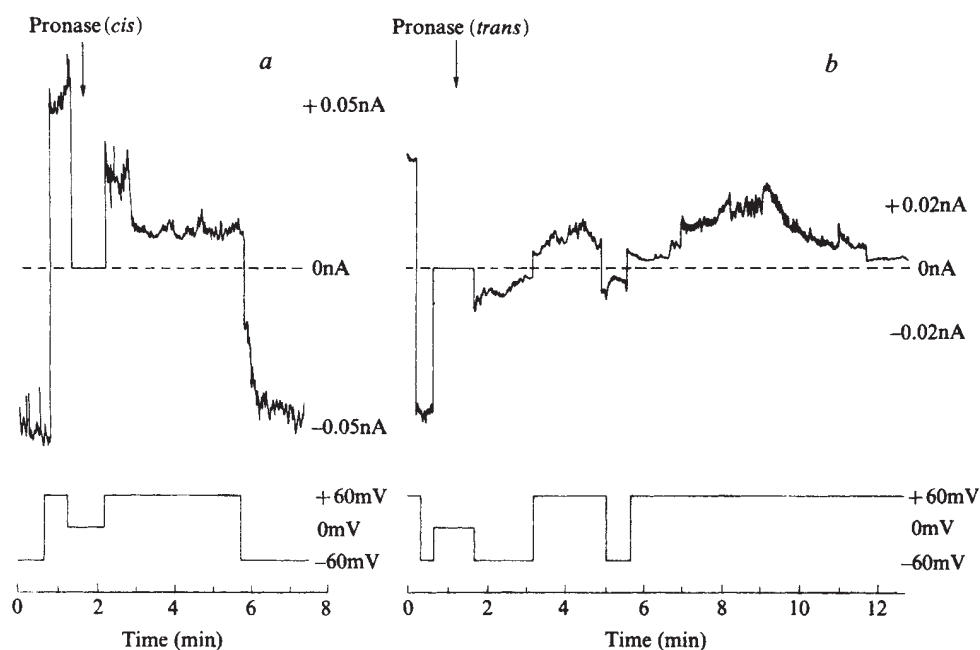


Fig. 3 The effect of pronase on the current through an HBP-ASOR-modified BLM. HBP and ASOR were added to the *cis* side. The conditions were as described in Fig. 2*b* legend. $100 \mu\text{g ml}^{-1}$ *Staphylococcus aureus* (Sigma) protease (equivalent to pronase) was added to the *cis* chamber (*a*) or to the *trans* chamber (*b*).

dependent penetration of the HPB into the vesicle bilayer. Valinomycin, in the presence of K^+ but without a K^+ gradient, had no effect on quenching.

The effects of ligand and Ca^{2+}

Figure 2 shows the pattern of conductance change induced in the BLM by HBP in the presence of the ligand asialo-orosomucoid (ASOR) and in the presence of Ca^{2+} . In each case the reagents were added to the *cis* side only. ASOR alone did not affect BLM conductance. With HBP alone (Fig. 2a), the conductance remained asymmetric, but it became symmetrical and increased rapidly when ASOR was added (Fig. 2b). To test the specificity of this effect, the terminal galactose residues of ASOR were removed by enzymatic hydrolysis with β -galactosidase. In accordance with the known specificity of the binding protein, the resulting product, agalacto-orosomucoid, failed to increase or symmetrize the conductance of an HBP-modified BLM.

In the presence of 2 mM Ca^{2+} (Fig. 2c) the conductance symmetrized, but the rate of conductance increase was relatively slow. Mg^{2+} had no effect. When ASOR (see Fig. 4) or Ca^{2+} (not shown) was added to the *trans* side of an asymmetrically conducting HBP-modified BLM, there was no effect on conductance and no symmetrization. As expected, addition of HBP alone to both sides of the BLM induced a symmetrical conductance (not shown). The simplest interpretation of the symmetrization seen with HBP and either ASOR or Ca^{2+} (Fig. 2) is movement of (part of) the HBP to the opposite side of the membrane.

Evidence for translocation of HBP across the membrane

We tested this notion of membrane crossing or 'translocation' by adding pronase to the *cis* side of an asymmetrically conducting BLM. This abolished the conductance increase, indicating degradation of HBP on that side; pronase had no effect when

added to the *trans* side. However, after conductance had been symmetrized by addition of HBP and ligand to a BLM, addition of pronase to the *cis* side resulted in an asymmetric conductance of the polarity corresponding to addition of HBP to the *trans* side of the membrane only (Fig. 3a). Thus with a *trans*-positive potential the conductance decreased. With a *trans*-negative potential the conductance initially increased, indicating that the HBP had entered a conducting state on the *trans* side. With prolonged exposure to *trans*-negative potential, all conductance eventually disappeared (not shown). This finding is consistent with a gradual potential-induced translocation of HBP back to the *cis* side, where it could be degraded by pronase. On the other hand, when the potential was maintained *trans*-positive, the protein was not degraded, and the new asymmetry could be maintained (not shown), indicating that pronase had not crossed the BLM.

When pronase was added to the *trans* side of a symmetrically conducting BLM (Fig. 3b), the pattern of conductance change was complicated but consistent with translocation of the conducting moiety of HBP. When the potential was made *trans*-negative, there was a decrease in conductance consistent with transition of HBP to a non-conducting state on the *cis* side and an inability to recruit conducting moieties from the *trans* side because those had been degraded by pronase. When the voltage was switched to *trans*-positive there was a transient increase in conductance due to transition of HBP into a conducting state, followed by a conductance decrease due to exposure of HBP which had apparently crossed over towards the pronase on the *trans* side. The conductance eventually disappeared when the potential was held *trans*-positive for longer times. Adding new HBP and ASOR to the *cis* side resulted in a repetition of the pattern.

Figure 4 suggests that a binding moiety of HBP is translocated. With HBP on the *cis* side, ligand had no effect when added to the *trans* side (Fig. 4a); when added to the *cis* side it increased and symmetrized the conductance (Fig. 2b). However,

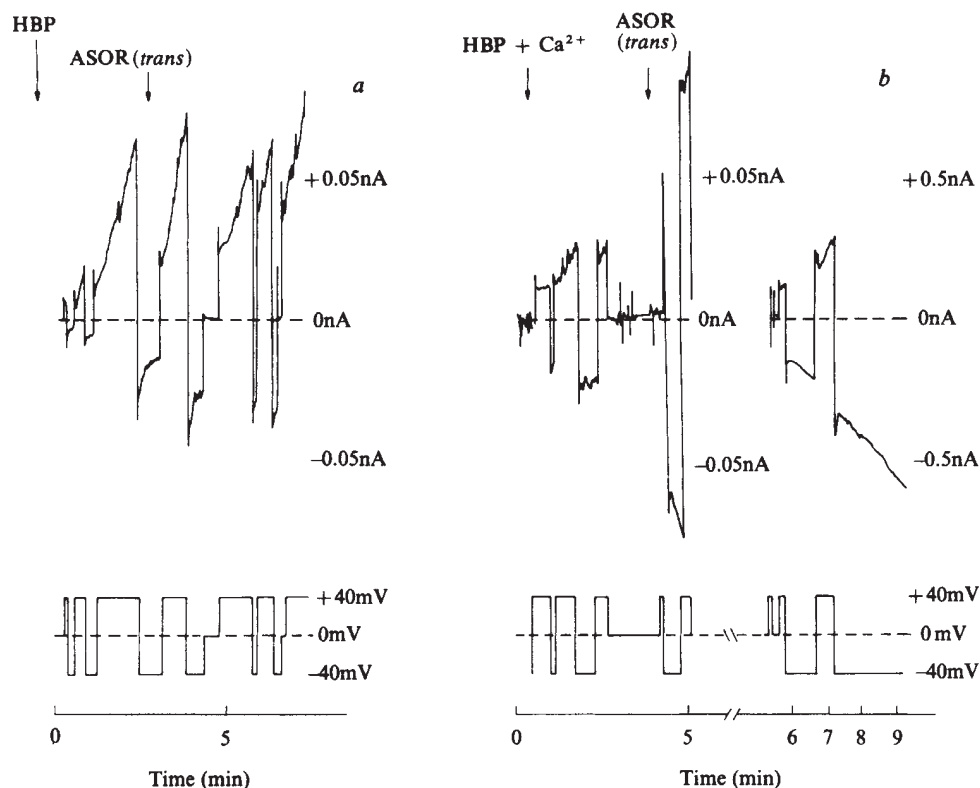


Fig. 4 Effect of adding ligand to the '*cis* and *trans*' sides. The conditions were as described in Fig. 1 legend. *a*, $10 \mu\text{g ml}^{-1}$ ASOR added to the *trans* side at about 2 min; *b*, 2 mM Ca^{2+} present on both sides and $10 \mu\text{g ml}^{-1}$ ASOR added to the *trans* side at ~3 min.

when a symmetrical conductance was induced in the presence of 2 mM Ca^{2+} , the conductance further increased markedly and symmetrically when ligand was added to the *trans* side (Fig. 4b). Again this was specific for the ligand; agalacto-orosomucoid had no effect on the conductance.

Figure 5 shows the absolute dependence of a conductance increase and subsequent symmetrization on the *trans*-positive potential. In the presence of HBP and ASOR a *trans*-negative voltage of 50 mV was applied across the BLM for 60 min giving no conductance change. When the voltage was then switched to a *trans*-positive polarity, the conductance started to increase and became symmetrical.

Discussion

Figure 6 shows a hypothetical view of the interaction between HBP and lipid bilayers. The protein first associates with the bilayer without requiring a membrane potential (a). Our previous studies⁶ have shown this to be hydrophobic interaction involving perturbation of the lipid bilayer and a conformational change in the HBP. Under the influence of a *trans*-positive membrane potential the protein can be promoted reversibly into a conducting state (b) in which it seems to perturb the bilayer sufficiently to permit passage of ions, that is, to increase the conductance of the membrane. The polarity of this effect makes sense, as HBP is negatively charged (isoelectric point at pH 4.7). The increase in conductance does not have the characteristics of a well defined channel or carrier. It does correlate, however, with the finding in lipid vesicles that HBP tryptophans become markedly less accessible to fluorescence quenchers in the aqueous phase when valinomycin and a K^+ gradient are used to generate a *trans*-positive potential. The conductance increase and the tryptophan sequestration suggest a further penetration by the protein, perhaps sufficient to perturb the second leaflet of the bilayer. However, these observations could also be accounted for by an aggregation, or by a combination of penetration and aggregation such as that in Fig. 6b. Analogously, Shinitzky has considered that vertical displacement of membrane proteins could be mediated by changes in lipid packing⁹.

The next stage (Fig. 6b, c) is a translocation of the protein from the *cis* to the *trans* side of the membrane under the influence of a *trans*-positive electric field in the presence of a galactose-terminal ligand or Ca^{2+} . Observations supporting translocation are (1) that the membrane conductance becomes symmetrical, (2) that part of the HBP becomes exposed on the *trans* side and is destroyed by pronase there, and (3) that ligand

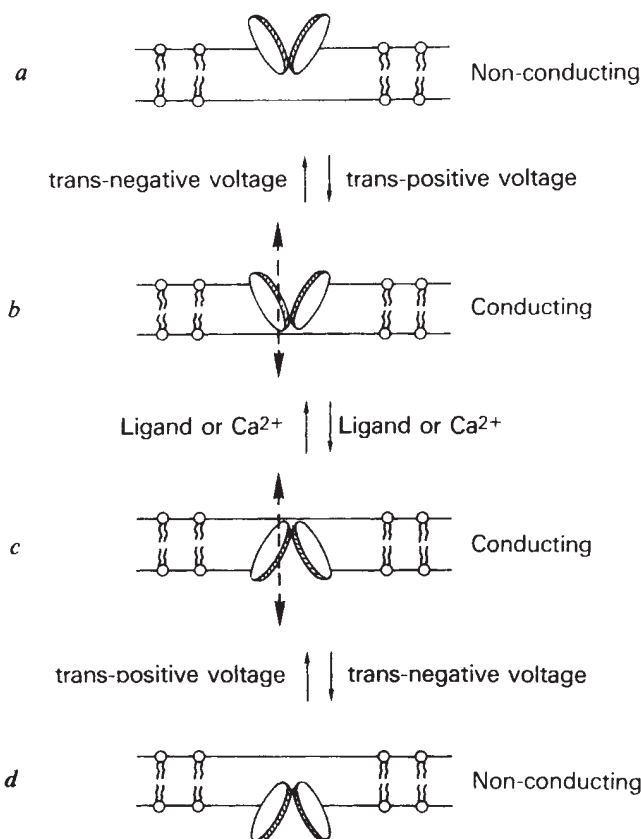


Fig. 6 Model for the translocation of the receptor protein (see text for explanation). The schematic rendering of two protein molecules is meant to suggest possible protein-protein interaction, not necessarily dimeric.

binding sites also become exposed on the *trans* side, where they are available for binding and cause a further increase in conductance. We do not know whether a given binding site exposed on the *cis* side becomes exposed on the *trans* side or whether an initially buried site is simply pushed through to the other side. Furthermore, we cannot say whether the protein actually flips over.

Two protein molecules are shown in Fig. 6 to indicate the possibility that protein-protein interaction is important in the translocation. It would provide an alternative to the energetically improbable movement of hydrophilic and charged residues directly through the low dielectric bilayer interior. Thus, charge delocalization and neutralization, by apposition of charged residues (either within a molecule of HBP or by aggregation as shown), could drastically lower the barrier. A possible role for Ca^{2+} in charge neutralization is suggested by the symmetrization seen in Fig. 2c.

Despite the topological problem outlined earlier, biochemical data suggest that the binding sites of HBP are on the cytosolic surface in the lysosomal fraction, and that they survive, presumably to be recycled to the plasma membrane. Although one must be cautious about the correspondence¹⁰ between biological membranes and a model system such as the BLM, our observations do provide a conceptual basis for the translocation that may be required. The hepatic cells are inside-negative¹¹, and the receptor is directed outward; measurements of ion distribution suggest that lysosomes are also inside-negative^{12,13}, and the receptor's binding sites are on the cytosolic, electrically positive surface.

There is considerable precedent for potential-dependent behaviour of peptides and proteins in membranes. Electrical conductance can be modulated asymmetrically by antibiotic ionophores¹⁴, toxins^{15,16}, transport molecules isolated from

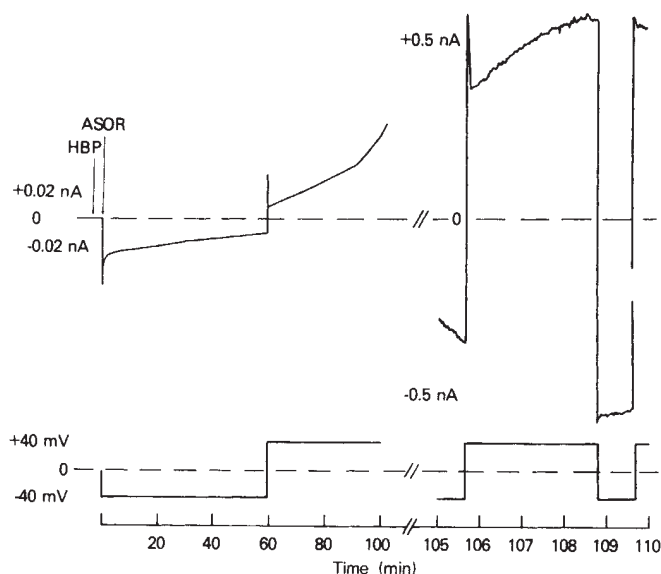


Fig. 5 Requirement of *trans*-positive potential for translocation of HBP across a BLM. The conditions were as described in Fig. 2b legend.

biological systems^{17,18}, immune cytotoxic factors^{19,20}, and by 'gating' components of the sodium and potassium channels of nerve^{21,22}. However, none of the physiologically interesting examples of these processes is understood in much detail. Wickner and co-workers²³ have speculated that an electrical gradient might drive newly synthesized M13 coliphage coat

proteins across the host membrane. This work on HBP provides the first direct evidence for the voltage-dependent translocation of a membrane protein across a membrane.

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The C1q receptor site on immunoglobulin G

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We propose that the binding site for the complement subcomponent C1q on immunoglobulin G involves the last two (C-terminal) β -strands of the C₂ domain. This region contains a large number of accessible and highly conserved charged residues and charge is postulated as an important component of the C1q-IgG interaction. The conclusions are reached on the basis of accessibility and sequence conservation analyses of C₂ amino acid residues, the use of specific inhibitors and chemical modification studies.

ANTIBODIES are the basis of the body's defence against infection. This central role is expressed in the functional versatility of the antibody molecule and realized through its unique structure. Thus, the organization of the antibody molecule into domains (Fig. 1a) allows recognition of a virtually unlimited range of antigens by the variable part of the structure, while the constant part mediates a small number of effector systems related to antigen elimination and/or immobilization. The best characterized of these effector systems is the classical pathway of complement.

Complement is a cascade process¹ generating a repertoire of biological activities with consequences² that are both local (for example, cytolysis and increased vascular permeability) and systemic (such as induced peripheral leukocytosis). Although the complement system presumably evolved as a beneficial antimicrobial mechanism, its inappropriate activation may lead to a variety of harmful consequences such as tissue damage secondary to complement-induced leukoembolization³, as occurs in adult respiratory distress syndrome, or complement-mediated cytolysis *in situ* such as occurs in autoimmune disorders².

The triggering event in the classical pathway is the binding of the first component C1 to the Fc region of aggregated immunoglobulin. C1 is a macromolecular complex of three subcomponents—C1q, C1r and C1s—of total molecular weight (MW) approximately 700,000. The binding site for the Fc region of Ig is found on the C1q subcomponent (MW ~400,000). The structure of C1q has been proposed by Reid and Porter⁴ as having the appearance of a 'bunch of tulips' (Fig. 1b). Eighteen chains, each some 200 amino acid residues long, are linked in threes in the base and stalk regions to form collagen-like triple helices. Each of the six fibres terminates in a globular head region which is thought to contain the Fc binding site(s).

The crystallographic structure of human Fc from pooled IgG

has been determined to 3.5 Å by Huber and colleagues⁵. The overall structure has been described as a 'Mickey Mouse', with the C₂ domains forming the ears and the C₃ domains the head (Fig. 1c). The tertiary structure of both these domains corresponds to the 'immunoglobulin fold'. However, whereas the pair of C₃ domains are in close interaction (Fab 'C-like' pairing⁶), the C₂ domains show no interaction with one another. The carbohydrate chains attached to Asn 297 are in between the C₂ domains. Although it is generally accepted that recognition of IgG by C1q occurs via a site on the C₂ domains^{6,7}, the precise location of the amino acid residues constituting this site has not been described.

We now propose an identification of the C1q binding site based on a novel approach containing three lines of investigation. These are: (1) amino acid residue accessibility and sequence conservation analyses which are used to locate potential binding regions on Fc; (2) specific inhibitors of the protein-protein interaction (IgG-C1q) which are investigated to give information on the nature of the groups involved in the interaction; (3) specific chemical modifications of residues on Fc and C1q which are studied to implicate particular types of residues in the protein-protein interaction.

The C1q binding site we propose is rather novel in that it consists mainly of charged side chains (glutamate and lysine) on the last two (C-terminal end) β -strands of the C₂ domain. The approach used may prove to be generally useful in studying protein-protein interactions, in particular, where a detailed structure is known for one of the proteins but little structural information is available for the second protein or the complex formed between the two.

The residues in the C₂ domain involved in interaction with C1q should fulfil two criteria. First, they should be accessible for C1q binding, that is, not buried in the interior of the domain. This can be quantified by the surface area of the residue which

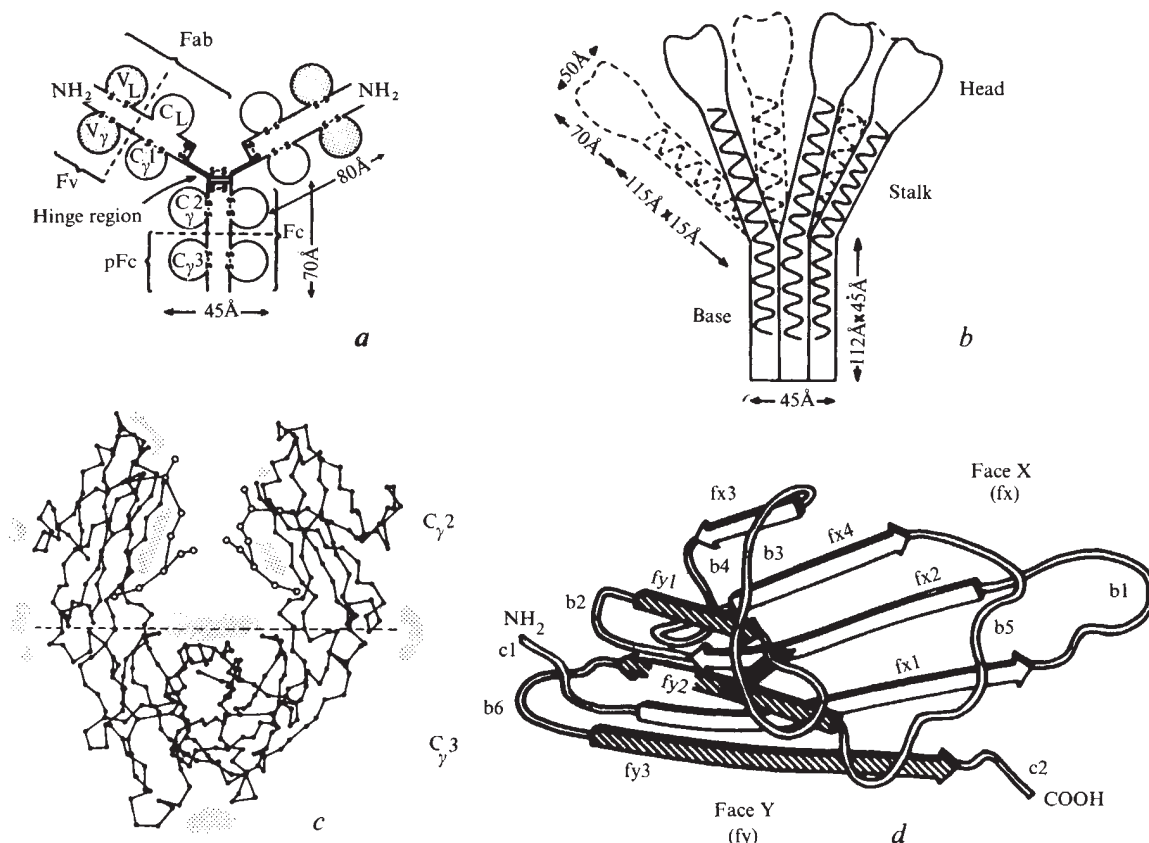


Fig. 1 *a*, The domain structure of IgG. The different domains are shown by circled regions where V signifies variable domain, C constant domain, γ the heavy chain and L the light chain. Various proteolytic fragments Fc, pFc, Fab and Fv are also indicated. The IgG molecules of different classes and species differ most noticeably at the hinge region. Human IgG1 is represented here. *b*, Molecular structure of C1q proposed by Reid and Porter⁴ (after whom the figure is taken) to account for the effects of collagenase and pepsin digestion and the dimensions and shape of the images seen in the electron microscope. Analysis and sequencing of the three types of polypeptide chain present show clear similarities between them, notably the repeating collagen-like triplets of residues which occupy some 85 positions starting near the N-terminal end. The characteristic Gly-X-Y pattern, where the residue Y is often either hydroxyproline or hydroxylysine, is broken at position 39 of the A chain and 36 of the C chain, allowing the bend of the triple helices forming each stalk away from those in the base region. *c*, The structure of the Fc fragment from pooled human IgG after Huber *et al.*⁵. $\bullet$, α -C carbon positions; $\circ$, approximate centres of carbohydrate hexose units. The very approximate locations of the main hydrophobic patches of the molecule (see Table 1 and text) are represented as shaded areas. *d*, Peptide chain folding of a constant domain⁶. The segments fx1-4 (unshaded) and fy1-3 form two roughly parallel faces of anti-parallel β -pleated sheet linked by an intra-chain disulphide bridge (filled rectangle, Cys 261 to Cys 321 in C γ 2). Between the β -pleated segments are other segments (b1-6) forming helices, bends and other structures. Segments fx3, fx4, fy1 and b4 are foreshortened in this three-dimensional representation. A generalized view of constant domain folding is represented here—the C γ 2 domain shows some deviations from this description, chiefly in b3 and fx3 (ref. 5).

can be in contact with the solvent. Second, they should be highly conserved in IgG molecules that bind C1q. This follows from the observation of the cross-species reactivity of C1q and IgG^{8,9}. These two criteria are now considered separately.

Accessibility analysis

In exploring those residues accessible for C1q binding, an analysis would ideally be carried out on the C γ 2 domain of IgG in an immune complex. However, the only structure available at sufficient resolution is that of the isolated Fc fragment determined by crystallography to 3.5 Å (ref. 5). In using this structure in the accessibility analysis, the assumption is thus made that Fc has essentially the same structure in the isolated fragment and in intact IgG. Further, it is assumed that antigen binding does not significantly perturb the Fc structure in IgG, in line with evidence from this and other laboratories¹⁰.

Starting with the crystallographic structure of the Fc fragment for which the positions of most of the atoms are known, those residues of the C γ 2 domain in contact with solvent can be identified using a computer program¹¹. This program calculates the solvent contact area by constructing the van der Waals surface of the protein and 'rolling' a sphere (in our case of 1.4 Å radius to represent a water molecule) over the surface. It is found that of the 104 residues of the C γ 2 domain, about 72 are accessible to water (contact area >5 Å²).

As the energy which drives protein-protein interactions often comes from shielding protein hydrophobic surfaces¹² (the area

buried being proportional to the free energy of the interaction, $1 \text{ Å}^2 \text{ contact area} \approx 80 \text{ cal mol}^{-1}$)¹³, first interest centres on accessible residues which are predominantly nonpolar. Groupings of residues to form exposed hydrophobic patches are of particular interest and the principal features of those located in the C γ 2 domain are summarized in Table 1 (see also Fig. 1c, d).

Table 1 shows that 35 residues have their nonpolar atoms (main chain α -C, side chain C excepting those attached to O or N and side chain S) accessible to solvent with a contact area >5 Å². Of these, 27 are clustered into four large patches (>100 Å²) (shown in Fig. 1) and the remaining residues are found in two smaller patches and as isolated residues.

The location of patches 1-3 makes it unlikely that they are involved in C1q binding (Table 1, Comments). Use of our second criterion that a potential C1q binding site should be conserved between species then helps to eliminate the remaining patches (Table 1, Conservation).

In the absence of suitable accessible hydrophobic residues the next stage is to examine hydrophilic residues. Most of these are, of course, solvent accessible and prime interest now centres on the conservation between species of these hydrophilic residues.

Sequence comparison analysis

Nine C γ 2 primary structures are available¹⁴⁻¹⁶: three human myeloma proteins [γ 1 (Eu), γ 3 (Zuc), γ 4 (Vin)], rabbit chain, guinea pig γ 1 and γ 2 chains, two mouse myeloma proteins [γ 1 (MOPC 21), γ 2a (MOPC 173)] and mouse γ 2b chain. Human

Table 1 The exposed hydrophobic patches of the C₂ domain of Fc from human IgG1

Patch	Approximate area (Å ²)	Residues	Conservation	Comments
Concave surface of the 4-strand β-sheet layer (x face)	150	Phe 241, Phe 243, Pro 244 Lys-Pro-Lys 246–248 Val-Val-Val 262–264	Very highly conserved. All residues, except Pro 247, identical in nine C ₂ primary structures	Mostly covered by Fc carbohydrate*. Lys-Pro-Lys is very close to C ₃ and probably inaccessible to C1q
N-proximal bend (b6)	130	Lys-Ala-Leu-Pro-Ala-Pro-Ile 326–332	Not highly conserved. Only Pro 329 and Ile 332 identical in nine C ₂ primary structures	Hinge nearby could increase hydrophobic area. In the case of antibody recognition of cell-surface determinants, C1q binding to this patch would be under severe, if not prohibitive, steric restrictions from the cell
C-proximal bulge (b1, b5)	100	Met-Ile 252–253 Val-Leu 308–309	Not highly conserved. Only Ile 253 identical in all nine C ₂ sequences	C1q binding to this patch would necessarily involve contact with C ₃ residues (compare with protein A ³²). The ability of the Fcαb fragment (IgG with the C ₃ domains removed) to bind C1 and activate complement by the classical pathway ⁷ argues against the involvement of this patch in C1q binding
Outside edge between β-sheet layers (fx3, fx4)	100	Val 279 Ala-Lys 287–288 Lys-Pro 290–291 Val 303 Leu 306	Not highly conserved. Only Ala 287 and Leu 306 identical in nine C ₂ primary structures	
C ₂ /C ₃ switch region	40	Ala-Lys 339–340	Ala 339 found in only four of nine C ₂ sequences	Relatively small area
b3 bend	40	Val 282, Val 284	Val 282 (most of nonpolar area) is identical in six of nine C ₂ sequences	Relatively small area
Isolated residues	Total area 60 Å ²	Pro 271, Lys 274, Tyr 296, Ile 336	Ile 336 highly conserved, others not	

* The crystallographic coordinates used in this analysis did not include those for the carbohydrate.

IgG1, IgG2 and IgG3, and rabbit and mouse IgG2a and IgG2b proteins are known to activate complement. Two mouse IgG1 subclasses—one activating complement, one not—have recently been proposed¹⁷. The C1q binding properties of MOPC 21 are not known. Guinea pig IgG1¹⁶ and human IgG4 proteins are known not to bind C1, although the Fc fragment of IgG4 does¹⁸. This latter observation has led to the suggestion that the C1q binding site may be present in the primary structures of all IgGs but unavailable in some cases because of, for example, obstruction by the Fab arms¹⁸. Furthermore, comparison of the C₂ sequences reveals no obvious correlation between primary structure and complement-fixing ability. Hence, all nine C₂ primary structures are considered in the detailed sequence comparison here.

The analysis reveals that of the 104 positions in the nine C₂ domains, 39 are identical throughout, and a further 36 may be considered conservative replacements (defined as any having a chemical relatedness index >0 according to McLachlan¹⁹; in the case of uncertainties, homology was maximized). Nineteen positions are particularly variable in that they show more than

one non-conservative substitution. As expected, these are almost exclusively surface residues and include two distinct regions: Gly-Val-Gln 281–283 and His 285 on bend b3 and Lys 288, Lys-Pro-Arg-Glu 290–294 on the fx3 sheet (nomenclature according to Fig. 1c). Interestingly, this latter region has been proposed as a C1q binding site by Brunhouse and Cebra¹⁶.

If we now consider the 39 invariant residue positions, some can be readily eliminated from being involved in C1q binding: 10 are buried, 5 are covered by carbohydrate and 2 (Asn 297, Thr 299) are related to sugar attachment. It then becomes necessary to establish positions for which invariance can be understood on structural grounds without invoking functional significance.

A comparison of the alignments of the primary structures of 23 constant domains²⁰ (including λ, κ, C_H1, C_H2, C_H3 and the 'extra' domains of various species) is now used to reveal whether any of the remaining 22 invariant positions are involved in general immunoglobulin domain architecture. The percentage of domains having the same amino acid residue as C₂ at the same position (the identity index) is computed. A high index

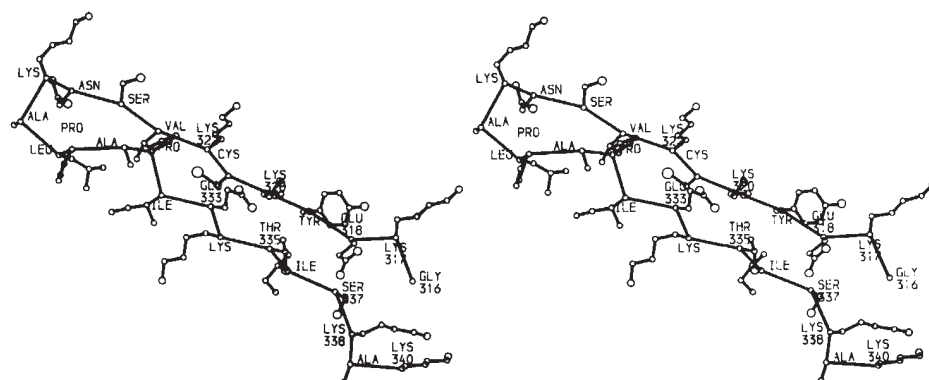


Fig. 2 A stereoview of the region of the C₂ domain containing the proposed C1q binding site. Residues 316 and 317 are part of a bend structure; residues 318–322 form a β-strand (fy2); residues 323–331 are on a bend as shown; residues 332–337 form a β-strand (fy3) antiparallel to fy2 and residues 338–340 continue into the C₃ domains.

indicates that the residue is important for the constant domain fold in general, a low index indicates it is unusual for constant domains and possibly associated with the unique function of the C_γ2. Table 2 shows identity indices for the 22 key invariant positions; Leu 306 and Trp 313 seem to be general features of constant domains.

At this stage, however, the most striking observation is the emergence of the contiguous residues on the γ face, Gly-Lys-Glu 316–318, Lys 320 (b5, fy2), Thr 335, Ser 337 and Lys 338 (fy3) (underlined, Table 2), as a potential binding region (Fig. 2). The charged residues have noticeably low identity indices. Further examination of other exposed residues in this region reveals that Lys 322 (fy2) is conserved as Lys in eight of nine C_γ2 domains, being replaced by Arg in guinea pig IgG2 and having an identity index of 13%. Pro 331 (b6) (replaced by Thr in guinea pig IgG1), Glu 333 (fy3) (Thr in guinea pig IgG1) and Lys 340 (e2) (Arg in rabbit), with identity indices of 35, 26 and 9%, are similarly conserved. The entire region (Fig. 2) is then one of exposed, highly conserved, mostly charged (five Lys, two Glu) residues.

Of the remaining residues of Table 2, Thr 260 and Arg 301 could be involved in interaction with the carbohydrate; Ser 239, Asp 265 and Ser 267 may be involved in hinge or Fab arm conformation; Lys 248 (possibly interacting with Glu 380) and His 310 are at the C_γ2/C_γ3 interface; Pro 329 is involved in bend architecture and Gly 341 in the C_γ2/C_γ3 switch region. Reasons for conservation of the remaining residues, Ala 287, Ile 253, Ile 332 and Ile 336, are unclear.

Note, finally, that the carbohydrate forms an exposed, probably conserved, continuous region and therefore must also be considered as a potential or part of a potential C1q binding site.

Inhibitor studies

In the light of the charged potential binding region on Fc discussed above, we have compared the ability of several salts, in a variety of experimental conditions, to inhibit C1q binding to anti-ovalbumin-ovalbumin aggregates. Table 3 compares the K_{50} values for a number of inhibitors.

Figure 3a illustrates the well known dependence of C1q binding to antibody aggregates on NaCl concentration^{21,22} and further reveals a marked anion dependence of inhibitory ability, $I^- > Cl^- > F^-$. Appropriate controls show that salt is not dissociating the aggregates over the concentration range used²³. Figure 3 also shows that the C1q-antibody aggregate reaction is reversible and therefore, at a given NaCl concentration, can be

Table 2 The identity indices of selected C_γ2 residues

	Identity index (%)	Total accessibility (Å ²)
Ser 239	30	16
Lys 248	13	28
Ile 253	10	38
Thr 260	30	11
Asp 265	35	22
Ser 267	22	11
Ala 287	4	21
Arg 301	4	30
Leu 306	87	26
His 310	4	34
Trp 313	65	7
Gly 316	35	11
<u>Lys 317</u>	22	23
<u>Glu 318</u>	17	24
Lys 320	4	15
Pro 329	17	40
Ile 332	15	13
<u>Thr 335</u>	35	21
Ile 336	13	10
<u>Ser 337</u>	30	12
<u>Lys 338</u>	13	6
Gly 341	38	13

Underlined residues are those on the γ face, forming a potential binding region (see text).

Table 3 Comparison of the ability of a number of molecules to inhibit the C1q-antibody aggregate reaction

Molecule	K_{50} (mM)	Molecule	K_{50} (mM)
Inorganic salts		Polyions	
NaSCN	170	DNA*	0.0005
NaI	200	Polylysine	<0.1
NaCl	260	Suramin	3.5
Na ₂ SO ₄	450	Heparin	1
NaF	>450		
NaI	150	Carbohydrates	
KI	175	Mannan	60
RbI	200	N-acetyl glucosamine	450
MgCl ₂	145	Raffinose	575
CaCl ₂	165	Xylose	575
CsCl	300	Glucose	>600
		Mannose	700
Organic ions		Glucose-6-phosphate	>19
N-(p-nitrophenyl)-2-aminoethyl phosphate	2	Lactose	>18
N-(DNP)-2-aminoethyl phosphate	2.5	Inulin	>225
DNP-lysine	2.5		
DNP-glycine	12.5		
DNP-leucine	>22.5		
DNP-OH	>10.5		
2,4-dinitronaphthol-7-sulphonate	2		
ATP	10		
ADP	9		
MnATP	11		
AMP	13		
Adenosine	>12		
Diaminobutane	55		
Spermine	25		
Glycine	>50		

K_{50} indicates the concentration of inhibitor at which C1q binding to antibody aggregates assayed as described in Fig. 3 legend is reduced by 50% from the maximum value (compare with Fig. 3a). For the inorganic ions, NaCl was present in concentrations such as to maintain a constant ionic strength of 170 mM. For the polyions and carbohydrates, the NaCl concentration was constant at 170 mM. > Indicates a full inhibition curve was not obtained and the value quoted is an extrapolation. >> Indicates that no inhibitory effect was observed up to the quoted concentration. For polylysine an upper limit is described because of uncertainties in the molecular weight of this polymer.

* NaCl = 120 mM.

described as an equilibrium process:



where AbAg represents antibody aggregate, measured in units of antibody concentration, and C1q·AbAg the complex, measured as the amount of C1q bound to the aggregates. Binding studies at variable C1q and NaCl concentration (Fig. 3b) can then be used to estimate a phenomenological dissociation constant, K_{obs} , at each NaCl concentration for this equilibrium. The plot of $\log K_{obs}$ against $a_{\pm}$ ($a_{\pm}$ defined as $a_{NaCl}^{1/2}$) in Fig. 3c reveals that K_{obs} increases with increasing NaCl concentration. This increase, together with a fall in C1q binding capacity, is responsible for the inhibitory effect of NaCl seen in Fig. 3a. The gradient of a $\log K_{obs}$ against $\log a_{\pm}$ plot can be interpreted in terms of a general equation for the effect of an electrolyte on a macroion equilibrium²⁴. Although a detailed analysis is probably not justified by the limited scope of our data, the clearly positive gradient of the $\log K_{obs}$ - $\log a_{\pm}$ plot indicates that ions participate directly in the association of C1q and antibody aggregate. The most straightforward interpretation of the slope is that a net total of 12 ± 2 ions are released when 1 molecule of C1q binds to antibody aggregates. A similar plot for CaCl₂ between 22 and 50 mM gives a slope of 15 ± 2 . Although both cations and anions could be involved, some indication of the importance of anions is provided by the dependence of inhibitory ability on the lyotropic series (Fig. 3a, Table 3), $SCN^- > I^- > Cl^- > F^- > SO_4^{2-}$ and the relative potency of organic

anions such as dinitrophenyl (DNP) ethyl phosphate and ATP as inhibitors (Table 3). It is interesting that the K_{50} values observed for NaCl (~250 mM) and ATP (~10 mM) are comparable with the binding constants to the organic phosphate site of oxyhaemoglobin of ~50 mM (NaCl) and ~2.5 mM (ATP), respectively²⁴. The inhibitory ability of organic ions listed in Table 3 may explain the widespread observation of various polypeptides (all containing negative charges) as inhibitors of the C1q-IgG interaction^{25,26}.

That the interaction between C1q and antibody-antigen aggregates involves ions does not, of itself, rule out the possibility that other interactions are important. In particular, the Fc carbohydrate could be involved because it fulfils the criteria of accessibility and conservation. However, neither monosaccharides (Table 3) nor the carbohydrate isolated from transferrin (similar to the human Fc carbohydrate) had any significant inhibitory ability²⁷. The inhibition by heparin is probably due to its polyionic nature.

Chemical modification studies

The effect of chemical modification of a number of groups on both C1q and rabbit IgG on the interaction between the two has been studied. Table 4 shows that modification of 19 ± 1 (30%) of the lysines on IgG by methyl acetimidate, a mild reagent leaving the positive charge on lysine intact, produces no decrease in the binding capacity of IgG aggregates for C1q, with a significant increase in the dissociation constant. A qualitatively similar

result is obtained for the modification of 4 or 10 lysines using acetic anhydride. A decrease in binding capacity and an increase in K_d are observed for modification of 12 ± 2 carboxyl groups on IgG by glycine methyl (or ethyl) ester (Table 4). (A recent report describes a similar observation of the effect of carboxyl modification on the C1q-human Fc interaction²⁸.) Control experiments in these and other modification experiments involved showing that: (1) circular dichroism (CD) spectra and (2) precipitin curves of modified and unmodified IgG were the same. A further control for methyl acetimidate modification of lysines and cyclohexanedione modification of arginines (see below) was that similar effects were observed whether the modification was carried out before or after antigen aggregation.

In contrast to the above, cyclohexanedione modification of 10 ± 1 arginines in IgG, of which 7 or 8 were estimated to be in the C_γ2 domains, has little effect on C1q binding, as shown in Table 4. The number of residues modified in the C_γ2 domains was determined from pepsin digestion of modified IgG as the difference between the number modified in intact IgG and the number modified in the F(ab')₂ and pFc' fragments²⁹. Six of the seven C_γ2 arginine residues in rabbit IgG are found in the general region between x and y faces. As the modification described involves complexing a very bulky cyclohexanedione-borate group to each arginine, our results provide evidence against the involvement of this region in C1q binding, which includes the region proposed by Brunhouse and Cebra as a C1q binding site¹⁶. This conclusion is also substantiated by the absence in rabbit from this region of the C_γ2 domain of lysine

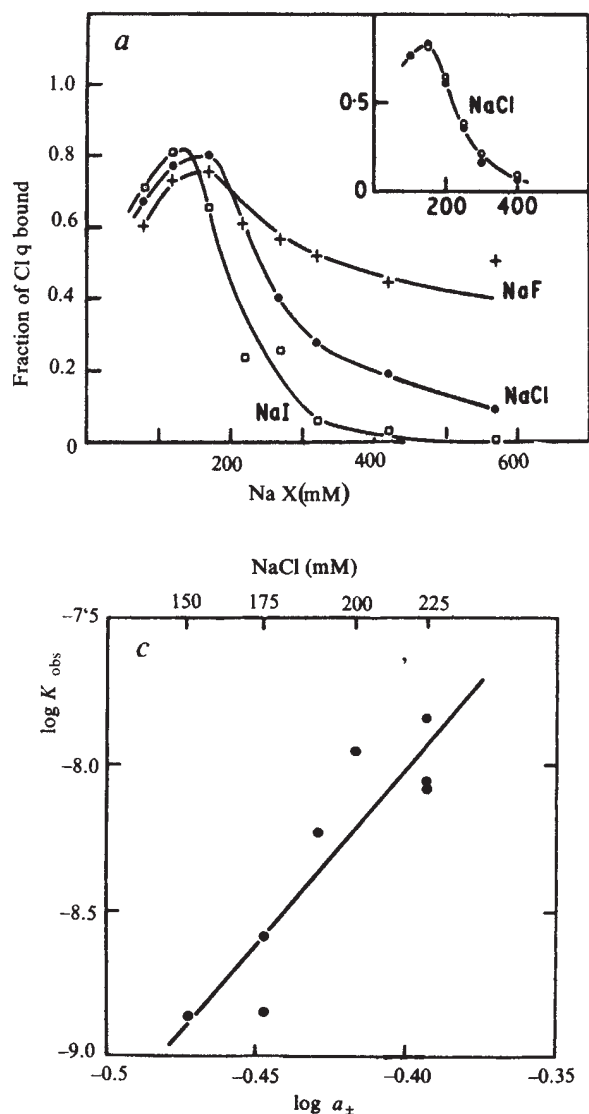


Fig. 3 The involvement of ions in the C1q-antibody aggregate reaction. *a*, The binding of C1q to antibody aggregates as a function of sodium halide concentration. Human C1q (ref. 38) and ^{125}I -labelled human C1q (ref. 39) were prepared as described elsewhere. Rabbit antisera were prepared by the injection of ovalbumin, emulsified in Freund's complete adjuvant 1:1, into each hind footpad and shoulder muscle. The anamnestic response was elicited by intraperitoneal injection of alum-precipitated ovalbumin. Purification to yield IgG was according to ref. 40. The C1q binding assay involved incubating ^{125}I -labelled C1q (typically $1\text{--}30 \mu\text{g ml}^{-1}$) with antibody aggregates (typically $25 \mu\text{g ml}^{-1}$) in a 12 mM Tris, 0.15% gelatin, pH 7.2, solution of the appropriate salt concentration at 37°C for 45 min. Bound and free ^{125}I -labelled C1q were determined after centrifugation. The inset data points were obtained as normal (●) or (○) by incubating as above for 30 min at 400 mM NaCl, performing the appropriate dilution to the specified NaCl concentration, reincubating and assaying in the normal manner. *b*, Representative binding curves for the reaction of C1q and antibody aggregates at varying NaCl concentration. Binding was measured as in *a*. The curves were fitted to a hyperbola using a nonlinear least squares program. The program yielded a value for K_{obs} , the phenomenological dissociation constant for the reaction, at each NaCl concentration. It was found that 10–20% (depending on the C1q preparation) of the C1q was not available for binding to antibody aggregates (possibly through denaturation). The binding curves shown have been appropriately corrected. *c*, $\log K_{\text{obs}}$ as a function of the log of the ion activity parameter, $a_{\pm}$, for the C1q-antibody aggregate reaction in the presence of NaCl. $a_{\pm}$ is defined as the square root of the NaCl activity ($a_{\text{NaCl}}^{1/2}$). The plot makes use of the type of data seen in *b*. The slope of the plot is 12 ± 2 .

Table 4 The effect of various chemical modifications of groups on IgG and C1q on the interaction between C1q and antibody aggregates

Modification	Extent of modification (per IgG or C1q molecule)	K_d modified K_d unmodified	Capacity modified Capacity unmodified
Modifications to IgG			
Lysine			
Methyl acetimidate	19 ± 1 (30%) 40 ± 5 (60%)	3–4	1
Acetic anhydride	4 ± 1 (6%) 10 ± 2 (15%)	2 4–5	1 1
Arginine			
Cyclohexanedione	10 ± 1 (30%)	1	0.8
Carboxylate			
Glycine methyl ester/ carbodiimide	4 ± 1 (3%) 12 ± 2 (10%)	1 2	1 0.5
Glycine ethyl ester/ carbodiimide	12 ± 2 (10%)	3–4	0.7
Tryptophan			
Hydroxynitrobenzyl bromide	1.5–2	1	1
Modifications to C1q			
Lysine			
Methyl acetimidate	95 ± 15 (75%)	1	1
Arginine			
Cyclohexanedione	18 ± 1 (10%)	—	0

For each modification a C1q binding curve is obtained (compare with Fig. 3b) and compared with unmodified reactants. The K_d and capacity of IgG for C1q in each case were determined from the binding curves as described in Fig. 3b legend. Methyl acetimidate modification³³ of lysines on IgG (10 mg ml⁻¹) was carried out at pH 8.06 and 4 or 25 °C in 0.1 M triethanolamine using 0.1 M methyl acetimidate for 3 min (30% modification) or 10 min (60%). The reaction was stopped by lowering the pH to below 8.0. Similar conditions were used for modification of C1q with 7 min modification time and the inclusion of 150 mM NaCl in all buffers. Acetic anhydride modification³⁴ of lysines was carried out at pH 5.1, 0 °C in 1 M sodium acetate using 0.5% (v/v) acetic anhydride for 5 min. The reaction was stopped by adding a large excess of lysine. The extent of lysine modification for both modifying reagents was determined using trinitrobenzene sulphonate as described elsewhere³⁵. Arginine modification^{29,36} was carried out using 1 M cyclohexanedione in 0.2 M sodium borate at pH 9 and 25 °C for 1 h. The reaction was stopped by lowering the pH to 4 and the extent of modification estimated from amino acid analysis. Similar conditions were used for modification of C1q with 45 min modification, a lowering of the pH to 6 to stop the reaction and the inclusion of 150 mM NaCl in all buffers. Carboxylate modification³⁵ of IgG made use of a 30–50-fold (over IgG) molar excess of carbodiimide in 1 M glycine methyl (or ethyl) ester at pH 4.75 and 25 °C for appropriate times. Excess sodium acetate was added to stop the reaction and the extent of modification estimated from amino acid analysis. Tryptophan modification³⁷ of IgG was carried out using a 300-fold (over IgG) excess of hydroxynitrobenzyl bromide in 0.1 M sodium acetate at pH 4.5. The extent of modification was estimated spectrophotometrically. Modifications were carried out on IgG in the range 10–70 µM.

residues which are shown here to be important in the interaction with C1q.

In sharp contrast to IgG, cyclohexanedione modification of 18 arginines on C1q (equivalent to 3 residues modified per C1q monomer unit) abolishes binding to IgG aggregates whereas methyl acetimidate modification of 75 ± 15% of the lysines on C1q has little or no effect.

Conclusions

The accessibility and conservation analyses described strongly suggest that a charged region on the last two (C-terminal end) β -strands of the C₂ domain of IgG has functional importance. Specific inhibitor and chemical modification studies are consistent with this function being the binding of the complement subcomponent, C1q, and we therefore propose that this charged region (Fig. 2) contains residues which are directly involved in the C1q binding site on IgG.

At the other end of the antibody molecule, recognition of antigen generally involves binding of the antigen in a cleft or groove in the antibody structure. On the basis of our hypothesis, recognition of C1q would involve quite different principles. Thus, the Fc site proposed by us consists of a virtually planar array of (mostly charged) residues which is unlikely to act as or bind into a cleft or groove. It is far more probable that recognition involves some form of surface 'matching' of charged

residues on Fc and C1q. This kind of protein-protein interaction involving charge has been described in the extended salt-bridge system between subunits in tobacco mosaic virus (TMV)³⁰. Whereas TMV uses residues on helices, our Fc site consists mainly of residues on two β -strands. A surface complementary to this would be provided by suitably arranged charged residues on two β -strands of C1q.

The type of approach we have adopted here is limited by the fact that it is not possible to describe the size of the proposed C1q binding site precisely. However, examination of Fig. 2 shows that Glu 318, Lys 320, Lys 322, Pro 331, Glu 333, Thr 335 and Ser 337 are fairly close in space and Gly 316, Lys 317, Lys 338 and Lys 340 (the residues not in the β -strands) are more distant. The former residues form a roughly planar area of approximately 150 Å². The location of this area in the C₂ domain is such that a bulky molecule such as a C1q head could be denied access for certain orientations of the Fab arms. In addition, our data do not definitively rule out the possible involvement of the contiguous carbohydrate moieties (compare with ref. 31).

Clearly, a crystallographic structure of Fc and C1q heads would provide a definitive identification of the C1q binding site. Progress in this formidable task could be made with the availability of a crystal structure for C1q heads. For instance, matching of this structure with that of Fc could be considered. In the short term, however, exhaustive testing of chemical modifications of Fc may allow selective labelling of particular residues in the site which can be identified by the usual protein chemical methods. Alternatively, crystallographic studies of Fc in the presence of an inhibitor could be useful. Such chemical modification and crystallographic studies are now in progress.

Finally, it is interesting that preliminary results on IgM (in collaboration with A. Feinstein) again indicate the importance of charged groups in the recognition of C1q. It remains to be shown whether the same site on C1q is recognized for both IgG and IgM.

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LETTERS

Prominent VLBI cores in powerful radio sources with arc second structure

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VLBI and aperture-synthesis techniques have produced better understanding of the structural details of extragalactic radio sources of both compact (<1 arcs) and extended (>10 arcs) types. Much less is known about the radio morphologies of sources with an overall size of just a few arc seconds, though such information is essential for understanding the evolutionary link between compact and extended sources. It has been predicted that the radio nucleus of a double source should appear Doppler brightened due to relativistic beaming, if the source axis is oriented close to the line of sight^{1–3}. To investigate this, we examined two samples of double radio sources whose axes are expected to be inclined respectively at large and small angles from the line of sight. The statistics of the 'core fraction'—fraction of the total flux of the source which is contributed by its nuclear core at some reasonably high frequency, say 5 GHz, can then be obtained. Sources with large inclination angles would probably dominate the samples of extended, well-resolved double sources and hence their core-fraction can be readily estimated from published aperture synthesis surveys. Double sources oriented close to the line of sight are expected to be more common in samples containing only those sources whose angular sizes are a couple of times smaller than the median size for that flux density range. A large, unbiased sample of this type can be constructed using the nine Ooty lunar occultation lists (see ref. 4). We have selected all sources lying north of declination -25° for which a flux density ≥ 1 Jy and an overall size between 1 and 4 arcs have been estimated at 327 MHz in the occultation observations. These 30 'few-arc second' sources are expected⁵ to be distant objects ($z > 0.3$) and hence intrinsically powerful radio emitters radiating $>10^{33}$ erg s⁻¹ Hz⁻¹ at 327 MHz ($H_0 = 50$ Km s⁻¹ Mpc⁻¹, $q_0 = 0.5$). Sources of such high radio power have a classical double morphology⁶ and there exists evidence for double structure in 8 of these 30 few-arc second sources. We report here a search for compact cores among these sources by VLBI at 5 GHz using the large antennas at Effelsberg and Westerbork which provide a minimum fringe spacing of 0.045 arcs at this frequency. Radio cores were found to be much more prominent in this representative sample of 30 few arc-second sources, as compared with the cores found typically in extended double sources.

The nine Ooty occultation lists provide high resolution data for an unbiased sample of 711 radio sources in the Moon's path, down to a flux level of $S_{327} \sim 0.25$ Jy. Due to the low frequency of this survey (327 MHz), it is believed that the sources have been selected without bias in orientation. Parts of these data have been used to study the angular size properties of weak

extragalactic radio sources^{5,7,8}. By excluding all sources with $S_{327} < 1$ Jy or $\delta < -25^\circ$, we first formed a sample A of 266 occultation sources, which should have properties very similar to the 4C complete sample (defined by $S_{178} > 2$ Jy). The median angular size⁵ of sources in sample A is ~ 12 arcs and three-quarters of them have been resolved in the occultation observations at 327 MHz (these low-frequency observations are suitable for measuring the overall size because the outer radio structure usually has a steep spectrum). The optical identification work for sample A has been carried out using PSS plates and using mostly a combined 'radio+optical' position accuracy of better than a few arc seconds, aided by the knowledge of radio structure. It was found that $\sim 39\%$ of the sources have optical counterparts brighter than the PSS plate limit⁴.

Our sample of 30 few-arc second sources was formed by selecting from the sample A all those sources for which occultation scans along position angles at least 20° apart had been observed and the derived maximum angular size at 327 MHz fell in the range 1–4 arcs. Note that a comparable number of similarly extended sources may have failed to be selected because, either the coverage in position angle during occultations was not wide enough or the angular resolution attained was insufficient to resolve them (the achieved resolution for the same source can vary by up to a factor of ~ 2 depending on the observational circumstances such as the speed of the Moon's limb relative to the source⁹).

The VLBI observations of the 30 few arc-second sources were made on 11 and 12 January 1980 for 26 h, using the 100-m Effelsberg telescope and 11 of the 14 telescopes of the Westerbork array in total power mode. The latter provides at the zenith a beamwidth of 7 arcs (EW) $\times$ 10 arc min (NS) and is equivalent to an 83-m dish in collecting area. A central frequency of 4,996 MHz and right circular polarization were used.

For each source, up to three short observations each lasting 10–15 min were made, interspersed with calibration observations. The minimum fringe spacing was 0.045 arcs, increasing by up to a factor of 3 for the southern sources. We also measured, on the first day, the integrated flux densities of all these sources at Effelsberg by taking cross scans at the source positions (known to an accuracy of ~ 1 arcs).

The VLBI data recording was done using the standard MKIIc system with a bandwidth of 2 MHz. A hydrogen maser oscillator at Effelsberg and a rubidium oscillator at Westerbork provided the time and frequency standards. The cross correlation and further reduction of the data were carried out at the Max-Planck-Institut für Radioastronomie, Bonn.

The values of integrated flux densities S_T , calibrated on 3C161 and 3C286, are given on the scale of Baars *et al.*¹⁰. These have an r.m.s. uncertainty of $\Delta S_T(\text{mJy}) = [(5)^2 + (0.05 S_T)^2]^{1/2}$. The correlated flux density scale was calibrated using the sources 0J287, 2134+004 and 3C454.3 whose flux densities measured at Effelsberg during the observations were 2.7, 9.8 and 8.3 Jy at 5 GHz, respectively. The calibration factor for all three sources has an r.m.s. scatter of 7%. Using the observations of these strong calibration sources and of a few weaker sources belonging to our observed sample (Table 1), the loss of correlated amplitude due to phase drifts in oscillators was determined as a function of the coherent integration time. Accordingly, a correction of 20% to the correlated amplitude was applied for a

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Table 1 Observed parameters of the 30 'few-arc sec' sources*

IAU name	Optical field†	Structure‡ and size at 327 MHz (arc s)	S_{327} (mJy)	$S_{4,996}$ (mJy)	$\alpha_{327}^{4,996}$	VLBI parameters at 4,996 MHz				
						PA (deg.)	FS (arc s)	S_c (mJy)	C_5	Class
0011+054	EF	D (3.5)	4,200	360	0.90	-47	0.049	<30	—	—
0057+105	EF	S (1.7)	1,000	168	0.65	-26	0.057	<30	—	—
						39	0.051	<30		
0352+210	18 mag NSO	S (2.7)	3,300	328	0.85	-24	0.051	94	0.31	<i>a</i>
						45	0.047	107		
0405+258	EF	S (2.0)	1,000	107	0.82	-25	0.049	<30	—	—
						44	0.046	<30		
0410+266	EF	D (3.0)	2,100	98	1.12	-26	0.049	29	0.30	<i>b</i>
						42	0.046	30		
0500+270	Crowded	S (3.5)	1,200	164	0.73	-19	0.049	31	0.20	<i>b</i>
						43	0.046	36		
0632+189	EF	PD (1.5)	3,000	124	1.17	-04	0.055	34	0.26	<i>b</i>
						32	0.050	32		
						44	0.047	30		
0646+184	EF	S (3.0)	1,400	107	0.94	-04	0.055	31	0.29	<i>b</i>
						46	0.046	30		
0656+213	19 mag G	S (1.5)	2,400	123	1.09	-05	0.053	47	0.39	<i>a</i>
						45	0.047	50		
0706+261	20 mag RO	D (3.5)	2,500	454	0.63	-09	0.051	<30	—	—
						44	0.046	<30		
0710+241	18.5 mag NSO	S (3.6)	1,200	74	1.02	-16	0.051	<30	—	—
						39	0.047	<30		
0736+167	EF	D (4.0)	1,800	82	1.13	-14	0.055	<30	—	—
						30	0.052	<30		
0746+162	EF	PD (3.4)	1,400	135	0.86	-14	0.056	85	0.47	<i>a</i>
						28	0.052	50		
						37	0.049	56		
1055+018	18 mag QSO	D (1.5)	4,000	2,985	0.11	38	0.058	3266	0.97	<i>a</i>
						48	0.049	2546		
1123+012	20 mag G	PD (3.5)	1,200	190	0.68	-50	0.047	36	0.18	<i>b</i>
						29	0.064	32		
1159-060	EF	S (1.6)	1,900	175	0.87	-48	0.054	87	0.50	<i>a</i>
1304-101	EF	S (4.0)	1,400	105	0.95	-51	0.054	27	0.34	<i>b</i>
						-39	0.073	44		
1348-129	EF	EXT	3,500	280	0.93	-52	0.059	69	0.26	<i>a</i>
						-47	0.067	76		
1350-154	EF	S (3.0)	1,000	45	1.14	-43	0.082	36	0.80	<i>b</i>
1456-165	20 mag BO	S (3.0)	1,500	189	0.76	7	0.125	66	0.35	<i>a</i>
1632-199	EF	S (3.5)	1,000	67	0.99	-45	0.097	47	0.72	<i>a</i>
						37	0.113	50		
1924-193	Crowded	D (2.8)	1,100	80	0.96	-45	0.093	<30	—	—
2019-202	EF	S (2.0)	1,200	320	0.48	-47	0.092	280	0.88	<i>a</i>
						43	0.104	283		
2023-142	EF/20 mag G	S (2.7)	1,000	32	1.26	-46	0.074	<30	—	—
						41	0.082	<30		
2050-188	EF	D (2.6)	2,100	140	0.99	-49	0.084	<30	—	—
						43	0.095	<30		
2058-179	19.5 mag BSO	S (3.3)	3,500	325	0.87	-11	0.079	33	0.10	<i>b</i>
						41	0.095	30		
2243-032	19 mag BO	EXT	3,500	503	0.71	-40	0.060	144	0.29	<i>a</i>
						40	0.061	147		
2245-022	EF	D (3.5)	1,250	264	0.57	-46	0.053	50	0.20	<i>a</i>
						-42	0.058	57		
						38	0.062	48		
2257-048	EF	S (2.2)	1,500	193	0.75	-44	0.058	33	0.17	<i>b</i>
2335+031	18 mag BL Lac	S (2.0)	4,300	603	0.72	-46	0.051	108	0.17	<i>a</i>
						-31	0.059	95		

* The optical and the radio (327 MHz) data are taken from Joshi and Singal⁴ and the references therein.

† NSO, neutral stellar object; QSO, quasi-stellar object; BSO, blue stellar object; RO, red object; BO, blue object; EF, empty field; G, galaxy.

‡ D, double; S, single; PD, possible double; EXT, bright core with a faint extension of a few arc s.

typical observation of 9 min. The r.m.s. noise for a coherent integration over such a period is ~ 8 mJy. The formal error of the correlated amplitude S_c is the quadratic sum of this noise and $0.05 S_c$.

The results for the 30 few-arc second sources are summarized in Table 1, together with their parameters at 327 MHz, derived from the occultation observations, and their optical identifications. The VLBI results include the fringe spacing FS, position angle PA of the projected baseline and the correlated flux density S_c at 5 GHz for each observation. The 'core fraction' at 5 GHz (C_5) gives the ratio of the mean correlated flux density of a source to its integrated flux density. The last column gives the fringe detection class: *a* indicates all detections above 5 r.m.s. (12 sources) and *b* the detections between 3.5 and 5 r.m.s. in at least one observation (9 sources). We consider the above 21 sources to be clearly detected because in each case, the maximum correlated amplitude: (1) lies well above the noise and

in the correct frequency side band; (2) it lies within the same range of three delay channels in which the calibration sources were also detected; and (3) its residual fringe rate falls in a narrow range from -58 to -74 mHz which is similar to the value -68 ± 4 mHz observed for the calibration sources.

Further, such consistent behaviour in at least two observations was a prerequisite for calling a fringe detection positive in all those class *b* sources where the maximum correlated amplitude was only between 3.5 and 4 r.m.s. It can be shown¹¹ using random noise statistics together with the above checks that at most 1 of the claimed 21 detections can be spurious. As a further check, we again correlated the data after artificially introducing an extra time delay of $2.5 \mu\text{s}$, which is equivalent to looking at 'empty sky' because the delay is ~ 5 times the coherence time defined by the system bandwidth of 2 MHz. Of the available 70 sets of observations, in only one case was a peak found on the correlated output which could satisfy all our detection criteria

specified above. Again, this empirical result on 'empty sky' suggests that all the 21 detections in our sample are most probably real.

For most sources, the position angle of the projected baseline changed appreciably between the observations. However, the fringe spacing remained around 0.05 arc s for sources at positive declinations but varied between 0.05 and 0.125 arc s for the southern sources. Two noticeable trends from Table 1 are: the correlated amplitude of a source changed little with position angle and showed no definite tendency to increase with fringe spacing, and the core fractions of the southern sources are not higher than those of the northern sources, though the former were observed at considerably larger fringe spacings. These two points together strongly suggest that all the detected compact components are essentially unresolved even at the smallest fringe spacing used and hence they are smaller than ~ 0.02 arc s in size. We expect these compact components to be associated with the optical nuclei of the sources, as there is not enough evidence that such compact and prominent structures occur in the outer lobes of radio sources. For instance, Broderick and Condon¹² made a sensitive VLBI search at 430 MHz for components smaller than ~ 0.03 arc s in a large sample of 100 extragalactic sources, which also includes some few-arc second sources. They found such compact components, contributing a significant fraction ($>3\%$) of the total flux density, exclusively in those sources which either have a flat radio spectrum or a quasar identification and therefore probably also possess a prominent nuclear radio core. A similar absence of compact hot spots (≤ 0.02 arc s) in the outer lobes has been reported for some few-arc second sources, such as 3C225B and 3C309.1, for which sufficiently detailed observations are now available¹³⁻¹⁶. Note that in recent VLBI work¹⁷ on 35 3CR sources with a fringe spacing of 0.16 arc s at 1.4 GHz, compact components have been detected in the outer lobes of many double sources. However, whenever they were found to be sufficiently strong (emitting $>5\%$ of the total flux density), these components showed evidence of resolution by the 0.16 arc s beam. In contrast, the compact components found in the present observations at 5 GHz seem to be considerably smaller and their contribution to the total emission is generally much higher than 5% (see Table 1). It therefore seems more appropriate to suppose that our detected compact components in the few-arc second sources are associated with their nuclear regions.

Each of the 30 few-arc second sources in our sample is probably a distant object located at a redshift $z > 0.3$ (and hence a powerful radio source radiating $\geq 10^{33}$ erg s⁻¹ Hz⁻¹ at 327 MHz). This follows from the analysis of radio source properties by Swarup and Subrahmanya⁵ and is also indicated by the optical identifications of these 30 sources (Table 1). Excluding two that lie in crowded fields, seven are identified with blue-stellar objects and hence are probably distant quasars; 18 lie in empty fields and are likely to be galaxies at $z \geq 0.4$ (refs 18-21). The remaining three sources have galaxy-like counterparts but even the brightest (and probably the nearest) of them is an object of $m_R \sim 19$ which translates to $z \sim 0.3$. These relatively high redshifts of the few-arc second sources should not, however, be systematically any different from the redshifts of the remaining 236 sources (mostly of larger angular size) in the parent sample A. This is because the optical identification rate of 37% among the 30 few-arc second sources is nearly identical to the identification rate of 39% mentioned above for the entire sample A. Consequently, the emission wavelengths of the observed radiation should not be systematically different for the two sets of sources. Among the few-arc second sources, there seems to be no obvious correlation between the presence of an optical identification and of a bright radio nucleus (Table 1). We notice a similar trend in the published VLBI observations of a complete sample of 100 extragalactic radio sources selected from the BDFL catalogue¹².

Table 1 shows that in 15 of the total 30 few-arc second sources, the radio nucleus accounts for more than a quarter of the total flux density at 5 GHz (in fact, similarly dominant radio

nuclei may exist in a few more of the 30 sources but these would have escaped detection because the sources are so weak that their total flux densities lie well below 100 mJy at 5 GHz). The above result on the fraction of sources with a core fraction $C_5 > 1/4$ can be parametrized by $F_5(>1/4) \geq 15/30 = 0.5$ and below we compare it with the corresponding values found for four fairly large and well-studied representative samples of extended radio sources again derived from metre wavelength catalogues.

(1) Sample 1 (galaxies): at NRAO, Bridle and Fomalont²² have mapped a large sample of 4C sources identified with galaxies brighter than 18 mag (double or triple morphology was commonly observed). Considering only those 150 sources with size >20 arc s and lying at $\delta > 15^\circ$, we estimate that a sub-arc s component with a core fraction $C_5 > 1/4$ at 5 GHz occurs in only 12 sources. This gives $F_5(>1/4) = 0.08$.

(2) Sample 2 (galaxies and quasars): in the 3CR complete sample, 66 sources show a classical double morphology with an established optical counterpart²³. In only two of them, is a core fraction $C_5 > 1/4$ seen²⁴. Thus, $F_5(>1/4) \sim 0.03$.

(3) Sample 3 (quasars): at Westerbork, Miley and Hartsuiker²⁵ have mapped at 5 GHz a sample of 114 4C quasars. Of them, 43 are classified as double with a size >20 arc s and possibly having a compact radio core which is clearly distinguishable from the straddling outer lobes. Of these 43, only seven have $C_5 > 1/4$. Thus, $F_5(>1/4) = 0.16$.

(4) Sample 4 (quasars): at NRAO, Potash and Wardle²⁶ have mapped a sample of 52 4C quasars at 2.7 and 8.1 GHz, of which 16 are larger than 20 arc s and show a double morphology with a possible central radio core. From these data, we estimate that three of the 16 quasars have $C_5 > 1/4$. Thus, $F_5(>1/4) = 0.19$.

The above comparison shows that in double sources of large angular size, whose axes are mostly expected at large angles from the line of sight, the radio nuclei are much less prominent as compared with those detected in the few-arc second sources by the present VLBI observations. In reality, the contrast is even more striking if one takes into account that the few-arc second sources were selected from a metre wavelength survey and hence $\sim 80\%$ are likely to be associated with galaxies (see above and refs 4, 18-21, 27), and that the radio nuclei in galaxies are an order of magnitude weaker than those in quasars of the same total luminosity (see ref. 28).

To understand fully the meaning of the very different levels of nuclear radio emission observed in the few-arc second sources and in sources of larger angular extent, one needs to assess the importance of projection in the former case. Generally, few-arc second sources can arise in two ways and depending on the relative strength of these two types among our 30, the present VLBI results can be taken as evidence for either or both of the following, each of which represents an extreme situation:

(1) If all the few-arc second sources are intrinsically normal sized doubles reduced to small angular size because of projection, the observed high core-fraction in them would be consistent with the hypothesis of beaming of the nuclear radio emission along the source axis¹⁻³. The implication of such beaming on the large scale structure of double sources has been discussed elsewhere²⁴. Note also that the occurrence of beaming in the nuclei of extended radio sources need not conflict with the opposite claim made recently for optically selected quasars²⁹, because the entire radio source in these quasars is visualized²⁹ as being so compact that the relativistic particles have yet to break out of the nuclear cloud of thermal plasma.

(2) Alternatively, the small angular size of few-arc second sources could be due to their being intrinsically small. If so, their physical sizes would only be ~ 10 -50 kpc but they would probably have a double morphology, as is typical for powerful radio sources⁶ (high radio luminosities for them were inferred above from their estimated large redshifts $z \geq 0.3$). Such an interpretation would assign to them properties radically different from what is known about powerful radio sources in nearby space, hinting that very strong cosmological effects would have to be present. For instance, at $z < 0.1$, intrinsic sizes³⁰ of

powerful double sources ($P_{1400} > 10^{32} \text{ erg s}^{-1} \text{ Hz}^{-1}$ for $H_0 = 50 \text{ km s}^{-1} \text{ Mpc}^{-1}$) are very rarely, if ever, $< 200 \text{ kpc}$. However, if the few-arc second sources are intrinsically small, the detection of very prominent nuclear radio cores in them would mean that the time scale over which the nuclear radio emission in powerful sources declines markedly, relative to the lobe emission, is comparable with the time needed by the lobes to emerge out of the main body of the parent galaxy.

To distinguish between the above two possibilities the extent to which projection determines their angular sizes needs to be assessed. Some insight into this question can be gained by mapping their arc second structures. Were the projection important, the two lobes might still be distinguishable but they would probably not appear elongated parallel to the line joining them. Note that such a morphology has been observed in the quasar 3C309.1 (a few-arc second source) mapped recently with a sub-arc second beam¹⁶, but is in contrast to the elongation of the lobes parallel to the source axis, as seen in extended double sources for which projection effects are expected to be minimal.

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Decrease in CO₂ mixing ratio observed in the stratosphere

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Air samples, collected cryogenically at different heights of the stratosphere, were analysed for carbon dioxide with an IR absorption technique. Supplementary tropospheric air samples were taken aboard commercial airliners. The results reported here show that the CO₂ mixing ratio is not constant with altitude but rather decreases in the stratosphere, by about 7 p.p.m.v., between the tropopause and 33 km. One conclusion is that recently increased concentrations of CO₂ in the troposphere have not propagated far into the stratosphere.

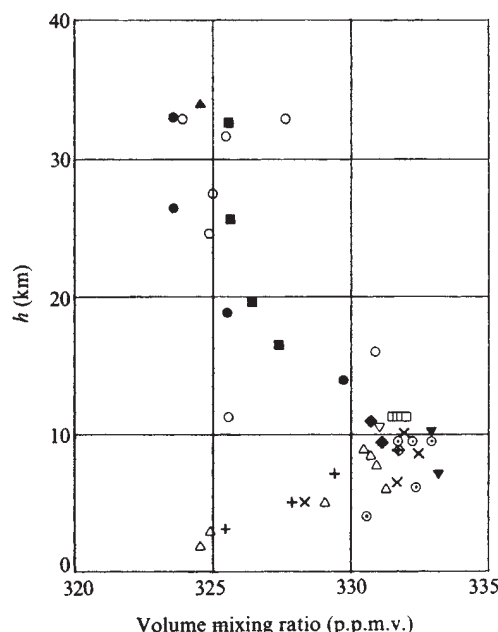


Fig. 1 Balloon and aircraft samples taken from 1977 to 1979. Balloon samples: ▲, September 1977; ●, June 1978; ■, 28 June 1979; ○, 9 November 1979. Aircraft samples 27 June 1979: ○, Stockholm; +, Copenhagen descent; ×, Copenhagen ascent; □, Zürich—Copenhagen; △, Zürich. Aircraft samples November 1979: ◆, Copenhagen, 12 November; ▼, Copenhagen, 15 November.

The balloon-borne cryogenic sampler is described elsewhere¹, together with mid-latitude, vertical profiles of H₂, CH₄, CO, N₂O, CFCl₃ and CF₂Cl₂ obtained from gas-chromatographic analysis of the samples. CO₂ can also be analysed by gas chromatography, but the total error is of the order of $\pm 1\%$, corresponding to $\sim \pm 3 \text{ p.p.m.v.}$ With the IR absorption technique, however, flask samples can be analysed with a total error of less than $\pm 0.2 \text{ p.p.m.v.}$, a figure which already includes the error from the absolute calibration².

For the first set of samples, taken during balloon flights in 1977 and 1978, which had been stored for more than one year, we found that some drifted considerably. In Fig. 1 we plot only the data from those samples which appeared to be stable—those that varied by less than 0.5 p.p.m.v. over a 3-month period. These clearly show that the stratospheric CO₂ mixing ratio is not constant with height but rather decreases with increasing height. This was confirmed by the results from samples collected on 28 June 1979. The CO₂ IR gas analysis was carried out 4 months after the flight. Although two samples whose CO₂ concentration drifted considerably were discarded, an apparently similar vertical profile was obtained from the stable samples. To demonstrate the vertical CO₂ distribution in the troposphere at the same time, supplementary air samples were collected on 27 June aboard commercial airliners, at different mid-latitude locations and different heights, and also analysed for CO₂. The results, also shown in Fig. 1, demonstrate that the highest CO₂ mixing ratios are observed at the tropopause level, which was at $\sim 10 \text{ km}$. The three stratospheric samples taken at 10.6-km altitude, during a Zürich—Copenhagen flight, suggest that the decrease in the CO₂ mixing ratio starts immediately at the tropopause. The decreasing CO₂ content from the tropopause level down, which is also noticeable in Fig. 1, is a result of CO₂ uptake by the vegetation at this time of the year.

The CO₂ mixing ratios analysed from seven stratospheric samples collected during a balloon flight on 9 November 1979, supplemented by five tropospheric samples from 12 and 15 November, fit fairly well with the vertical distribution of carbon dioxide, although some scatter is noticeable. Because no repeated analysis checks could be made for this last set of air

samples, we cannot decide whether or not this scatter is real or due to concentration drifts in some sample containers.

Our results show that the stratospheric CO_2 mixing ratio decreases from 332 p.p.m.v. at the tropopause to ~ 325 p.p.m.v. at a height of 25 km. These values are related to the Scripps 1959 manometric scale (see ref. 2). Our data do not give any indication of a vertical gradient higher up. This decrease of 7 p.p.m.v. disagrees with results obtained by Volz *et al.*³. They found, through gas-chromatographic analysis of stratospheric air samples, a constant CO_2 mixing ratio of ~ 320 p.p.m.v. for the whole height range discussed here.

Because the presently known photochemical reactions cannot account for the stratospheric CO_2 decrease we found, this is likely to be the result of long-range transport and exchange between the troposphere and stratosphere. In fact, due to the burning of fossil fuel, the tropospheric CO_2 mixing ratio increases by ~ 1 – 1.5 p.p.m.v. every year² and had an annual average for 1979 of 332 p.p.m.v. This man-made increase in tropospheric CO_2 is gradually propagating upwards with a delay that increases with increasing altitude. The vertical gradient shown in the diagram, which is a result of this slow vertical mixing, suggests a delay of 5–6 yr between the tropopause and an altitude of 25 km.

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Grand unification magnetic monopoles inside the Earth

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Elementary particle grand unification theories admit the possibility of very massive magnetic monopoles, but standard cosmology gives too many poles. Massive monopoles are affected both by gravity and magnetism, requiring reassessment of existing monopole searches. Monopoles inside the Earth would be able to annihilate with each field reversal, thereby giving rise to additional internal heat. This perspective is used to estimate an upper limit for the monopole-to-baryon ratio at accretion of 10^{-28} .

Grand unification particle theories such as $\text{SU}(5)$ allow the existence of massive magnetic monopoles¹. Polyakov and 't Hooft^{2,3} observed that theories such as $\text{SU}(5)$ with an imbedded $\text{U}(1)$ symmetry could contain a magnetic monopole solution^{4,5}. Goddard and Olive⁶ noted that this approach can reproduce Dirac charge quantization⁷ by using a massive vector boson. Bogomol'nyi⁸ has found a lower limit on the monopole mass of $M > 4M_v/g^2$ where M_v is the mass of the boson and g^2 is a dimensionless coupling constant. If M_v is the vector boson of weak interactions, g^2 is equal to α , and the Bogomol'nyi limit is of the order of 10^4 GeV.

Many grand unification theories are characterized by the presence of super heavy bosons with a mass^{9,10} of the order of $M_x = 10^{14}$ GeV. The mass of a grand unification magnetic monopole (GUMM) is set by the Bogomol'nyi limit with the related coupling constant of the order of $1/50$ (ref. 11), so that GUMMs are expected to have masses of the order of 10^{16} GeV or $0.02 \mu\text{g}$.

Such massive particles might have been produced in the big bang in the early Universe. It has been shown^{12–14} that for standard cosmology and plausible grand unification models that the number of GUMMs would be close to the number of baryons even now. The evidence is against this in our corner of the Universe. Several ways of circumventing Preskill's paradox have been suggested. These include first-order phase transitions¹⁵, electric charge non-conservation^{16,17}, unusual group structures¹⁸ and cosmological statistical non-equilibrium¹⁹. The grand unification–standard cosmology system is then faced with a paradox that can be moderated or even turned off but with difficulty. Grand unification remains to be established as a fundamental relevant theory and standard cosmology seems to leave some room for adjustments. However, this paradoxical situation means we need to understand better the limits on the existence of massive magnetic monopoles.

A massive magnetic monopole could be produced either in the big bang or in a cosmic-ray interaction. Bludman and Ruderman²⁰ have shown that it is not feasible to produce particles with the masses of GUMMs in cosmic ray collisions. A GUMM born in the primordial fireball that survives annihilation can be accelerated to 10^{11} GeV by galactic fields^{21,22} of the order of 3×10^{-6} G acting over distances of 10^{21} cm (ref. 23). As these are non-relativistic energies, previous conventional wisdom on energy loss of relativistic monopoles and on transit times must be used with discretion.

No magnetic monopoles have been found²⁴. In a typical search, magnetic charges are expected to produce heavy ionization in matter, but for non-relativistic energies the energy loss is smaller²⁵. Where timed counters are used, care must be taken. Typically²⁶ a pole accelerated for 50 cm in an 80-kg field and detected with counters spaced 25-cm apart and a timing window of 20 ns, would give a minimum β of 0.04 and an upper limit for mass that could be detected of 10^5 GeV.

A monopole passing through a coil should produce an induced voltage²⁷. This technique has been exploited to show that the monopole-to-baryon ratio is $< 3 \times 10^{-28}$ near the surface of the Moon²⁸. As with all other searches this limit is affected by the circumstances of the monopole trapping.

Parker has shown that monopoles would have a profound effect in dissipating and neutralizing large-scale magnetic fields. For relativistic Dirac poles this sets a limit on the monopole-to-baryon ratio for the galactic system of 2×10^{-26} , while for non-relativistic poles it would be around 10^{-24} . Solar and terrestrial field limits are less stringent and the galactic limit is less stringent than the lunar search.

A primordial magnetic monopole is subjected to forces from cosmological magnetic fields, gravitational forces, other magnetic monopoles, and interactions with ordinary electronic matter through processes such as ionization. Many analyses of pole behaviour only consider the magnetic effects. For GUMMs, the force of gravity will approach the effect of cosmic magnetic fields. The gravitational energy acquired by a typical GUMM falling into a galactic disk is 10^{10} GeV while the energy gained in passing through a galactic magnetic field fluxuation (300 light yr) is 2×10^{10} GeV. A GUMM with this energy could go deep into the Earth. The magnetic force at the galaxy is 500 times the gravitational force but the gravitational force acts over a much larger distance. The gravitational energy from solar or terrestrial sources exceeds the energy acquired from the corresponding magnetic field. For a magnetic dipole Earth acting in a repulsive sense (not a realistic approximation here) the magnetic and gravitational forces are in equilibrium at $R = 0.18 R_c$. This is well inside the radius of the core ($R_c = 0.55 R_e$).

In a dense material a GUMM will be thermalized and move to an equilibrium position of the magnetic, gravitational, and other forces. In a dipole Earth this would be on a line with the opposite terrestrial magnetic pole and the gravitational centre. The same is true for magnetic fields generated by currents. If the pole is in a fluid it will be able to move freely. In a solid it may have to move by lattice jumps²⁹.

Because gravitation forces and magnetic forces are intermingled, one must be cautious about where one looks for GUMMs. A heavy pole that accretes in equilibrium with the material of the Earth will move preferentially closer to the Earth's centre because of the presence of a terrestrial magnetic field. Thus, the Earth's surface is a poor place to look for GUMMs.

The Earth's magnetic field reverses irregularly^{23,30} with epochs lasting typically a million years interspersed with occasional events of shorter duration. The reversal takes of the order of 1,000 yr and is turbulent. The Earth's magnetic field is due to a convective dynamo in the fluid core. The dipole field lines flow into the core and then spiral around the Earth's axis of rotation, resulting in larger axial (100 G) than poloidal fluxes (5 G). Further complexity is introduced by convective cells within the dynamo.

When the field reverses, GUMMs of one polarity would move to positions previously held by the opposite polarity. Some of them would pass close enough to opposite sign poles to attract, bind and annihilate, resulting in the release of an extraordinary amount of energy. What, then, would be the contribution of such an energy source to the terrestrial heat inventory?

The heat generation inside the Earth is not completely understood³¹. Heat comes from both gravitational forces and radioactivity. Differentiation processes concentrate radioactive materials selectively in the silicates which form the Earth's crust so that much of the radioactivity is near the surface. Estimates of average heat flow per unit area over the surface area have been revised upwards in the past decade by 20–40%. Uncertainties include the amount of radioactivity, thermal properties of the high-pressure core material, and the contributions of the ocean basins. Clearly, then, understanding the relevant contributions to this heat flow is no easy matter. The average heat flow at the surface is of the order of 1.5×10^{-6} cal cm⁻² s⁻¹. The total heat output is 3×10^{20} erg s⁻¹ (2×10^{23} GeV s⁻¹).

To estimate an upper limit on monopole annihilation as a heat source, we assume that of the order of 25% of the Earth's heat generation could easily come from this source. This corresponds to a heat source of 5×10^{22} GeV s⁻¹. For a field reversal every 500,000 yr each reversal must supply 8×10^{35} GeV. If the annihilation energy is perfectly coupled to terrestrial material this requires the annihilation of 10^{20} GUMMs. If the annihilation region is confined to the Earth's core the annihilation event density is $n_a = 4 \times 10^{-7}$ cm⁻³.

One model to get a monopole density for one polarity, n , is to assume that the core is uniformly filled with north and south poles. Poles are moving with some relative velocity v_D as they wander through the liquid core. When they approach within r_a where the pole–antipole force is sufficiently strong to overcome external forces, they will annihilate, provided the angular momentum between the poles is small enough. The annihilation event density is

$$n_a = \pi r_a^2 v_D t n^2 \quad (1)$$

where t is the time during which annihilation can occur in the reversal. Initial inhomogeneities would have to be present because the monopoles with opposite polarity would be separated. But, as the reversal proceeds, the equilibrium condition could exist over different smaller regions of the core. The model would still be satisfactory provided t represented the average time for some region to pass through equilibrium.

For a monopole in equilibrium with the liquid core at a temperature of 4,000 K, the thermal velocity is of the order of 10^{-2} cm s⁻¹, as is the average mass drift velocity. But for an axial field of 100 G, the characteristic monopole velocity might be 10^5 cm s⁻¹ based on extrapolating the Ahlen energy loss formula²⁵ to low β values. With the complex field geometry fully on, monopoles would move to equilibrium positions probably near the surface of the solid core or the inner surface of the mantle in ~ 1 yr. If the reversal is turbulent, though, monopoles might slow substantially for some period even as they moved to regions where field lines had moved in again. At a velocity of

10^{-2} cm s⁻¹ the time to produce a homogeneous condition is of the order of 10^{10} s.

The capture distance, r_a , is sensitive to gravity, the terrestrial magnetic field and relative pole velocity. For characteristic forces that would be present (10^{-5} dyne), a plausible capture distance would be 10^{-5} cm.

For these conditions, the monopole density in the core to produce one-quarter of the Earth's heat is $n = 1.5 \times 10^{-3}$ cm⁻³. This is equivalent to an upper limit on the monopole-to-baryon ratio at accretion of 2×10^{-28} . The baryon number is taken for the entire Earth rather than the core alone as it is presumed that monopoles were differentially attracted to the core. The pole density could not be too much larger because the poles would cancel the Earth's field. Field dissipation due to magnetic currents is less of a constraint.

This limit depends on the pole mass. Turbulence and inhomogeneities could also change the limit. It is difficult to attach any estimate of accuracy to a limit based on such a speculative mechanism. Nevertheless, the picture suggests that the upper limit on the monopole-to-baryon ratio in the Earth is extremely small and consistent with the upper limit on the ratio observed at the surface of the Moon. This strengthens the Preskill paradox and indicates that the grand unification–standard cosmology–GUMM collection picture must break down at some point.

A GUMM density near this limit would be sufficient to supply energy for of the order of 10^4 field reversals (the age of the Earth). Such a density would degrade the Earth's field by 10% with the details depending on the equilibrium configuration. For a constant dynamo strength over the life of the Earth the average net field would rise with time as the monopoles annihilated.

There has been little consideration of the details of annihilation of GUMM pairs but they would probably annihilate rapidly into many particles with energies of 10^{14} GeV, some of which would decay quickly into muons and neutrinos. Muons at these energies lose nearly all of their energy by radiation rather than ionization³³ so that the energy is dissipated within 20 km of the annihilation point. The neutrino cross-section³⁴ is expected to be of the order of $\sigma_\nu = 10^{-34}$ cm², so that a substantial fraction of the neutrinos would interact in the Earth. During a field reversal much of the GUMM mass would be converted to heat in the core. Heat transfer times from the core are of the order of the age of the Earth so that the individual field reversals would be averaged out. Some portion of the neutrino energy deposited in a layer close to the surface (roughly 10 km) would appear as an effective heat pulse during a field reversal. This is only 0.1% of the neutrino energy and substantially less of the total but appears in 1/500 the time so that it might constitute a thermal spike of the order of 0.01–0.1 of the average heat flow. This spike seems to be insufficient to have an effect on the polar ice caps³⁵.

The Moon has no dipole-like field³⁶, but a field of 0.05–1 g would have been necessary to produce the magnetization³⁷. It is difficult to model a lunar dynamo that would have produced the field²³. If the Moon accreted from a mix with a GUMM-to-baryon ratio five times larger than the upper limit earlier suggested for the Earth and the GUMMs differentiated out into opposite poles during the process their presence could explain a slowly decaying relic field of the order of 0.1 G. Some mechanism such as an external field from the Earth of the solar wind would have been needed to establish the north–south separation.

Thus even small admixtures of GUMMs would produce noticeable changes in planetary conditions. On the other hand the search for massive primordial monopoles should continue. Possibilities might include meteors from the cores of large asteroid bodies. Perhaps some reconsideration should be given to tracks in emulsion purported to come from one-third of Dirac charge monopoles in experiments on meteorites³⁸. These could have been due to massive, slowly moving monopoles with concomitantly smaller energy loss. A later experiment was more sensitive but not to GUMM masses.

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Authigenic dolomite on marble surface

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The formation of dolomite remains controversial. Various hypotheses have been proposed, including primary precipitation from seawater, authigenesis or penecontemporaneous alteration of CaCO_3 by seawater, and postdiagenetic secondary replacement of CaCO_3 by groundwater, connate water, or other solutions. Although the existence of authigenic dolomite in recent sediments has been confirmed¹⁻³, all attempts to synthesize ordered dolomite at temperatures of sedimentary environments have failed⁴. It has been suggested that organic material^{5,6} and clay minerals^{7,8} may be important in the formation mechanism of dolomite. Here we present evidence that authigenic dolomite is formed on marble surfaces exposed to urban atmospheres. This discovery suggests that a relatively short period of time (of the order of a century) is actually required for dolomite formation in the natural system described here.

We have studied for many years the effects of the atmosphere on the deterioration of building stones. Samples from San Petronio (fourteenth century) and other more recent buildings in Bologna (Northern Italy) have been examined. Analyses on many altered layers found on limestone and marble surfaces have shown the following parageneses:

- (1) authigenic dolomite and calcite, quartz, K-feldspar;
- (2) authigenic calcite, quartz, K-feldspar;
- (3) authigenic gypsum and calcite, K-feldspar, Na-feldspar, graphite;

(4) authigenic gypsum, quartz, K-feldspar, graphite. The thickness of the altered layer varies between <1 mm to several mm (Fig. 1).

The variation in the mineralogical parageneses of the altered layer is outlined in Fig. 2. The dolomite clearly prevails in the external layer (A) and decreases progressively from the outside towards the inside until it disappears on contact with the unaltered rock. In the samples where it is only present in traces it is exclusively observed in the superficial layer.

Representative X-ray diffraction analyses (XRD) of samples of calcite and dolomite are shown in Fig. 3. Our results indicate that: (1) in layers A, B, C and D the dolomite presents a slight excess of calcium¹² ($\text{Ca}_{53}\text{Mg}_{47}$) and the calcite shows a MgCO_3 mole content of 2%; (2) the calcite in the original marble has also a 2 mole % MgCO_3 ; (3) in the layer where only the calcite precipitates, that is in the sample of the second paragenesis, a high-Mg calcite ($\text{Ca}_{90}\text{Mg}_{10}$) is present. The error in the determination is <1 mole.

The XRD analyses of marble samples dissolved in a 2% solution of acetic acid show that dolomite is not present in the unaltered rock. Sedimentation of dolomite on the surfaces observed as wind-blown dust seems unlikely as then dolomite should also be found in the other parageneses as occurs for quartz and K-feldspar, which seem to be derived from soil erosion. This observation together with optical analyses which show euhedral or subhedral forms and intergrowth texture (Fig. 1) excludes this origin. Nearly stoichiometric calcite was observed alongside dolomite. This phenomenon together with the absence of magnesian calcites means that a metasomatic origin can be excluded. These observations lead us to think that the

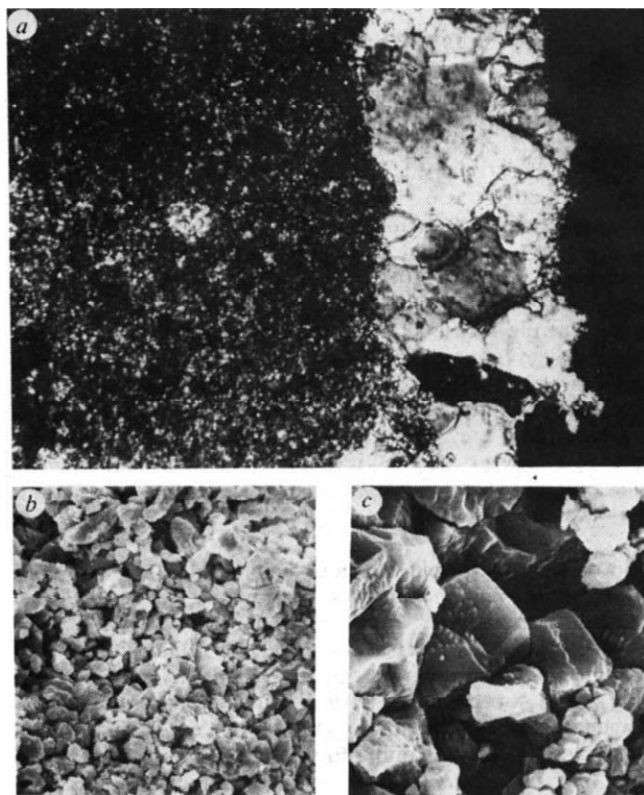


Fig. 1 The altered layer consists of rhombohedral crystals of dolomite and calcite, often twins, with greater dimensions (5–10 μm) than the crystals of calcite in the unaltered marble. The porosity of the altered layer is high. It is impossible to discriminate calcite from dolomite by optical analysis, but X-ray analyses show that dolomite prevails in the outer layer. a, Thin section, Nicols $\times$, $\times 100$; b, scanning electron microscopy (SEM), $\times 1,000$; c, SEM, $\times 3,000$.

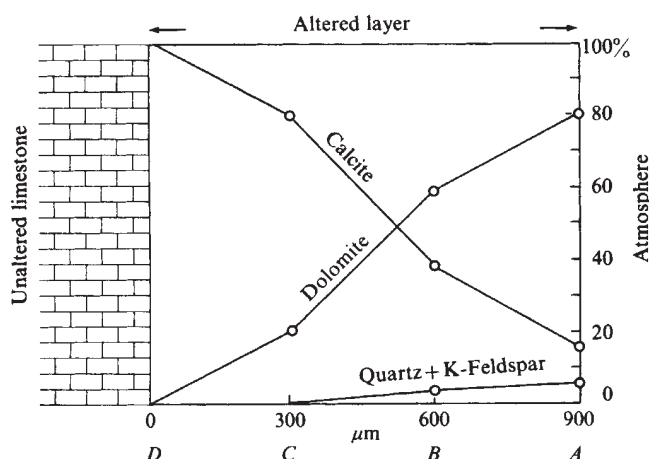


Fig. 2 Schematic diagram of the mineralogical concentrations of the altered layer. The altered layer is of an average thickness of 1 mm. For each area the samples were taken successively A, B, C, D and each were 200–300 μm thick. Quartz and K-feldspar percentages were determined after dissolution of the samples in acetic acid.

dolomite is formed by direct crystallization from aqueous solution. The unaltered rock contains between 0.25 and 0.30% Mg. We think that the Mg in our system does not derive exclusively from the dissolution of the underlying rock, but also from the washing away of the surrounding area. Besides, there is evidence⁹ (even though our thermodynamic and chemical conditions differ) of a mobilization of the Mg ion in the solid state, which could affect a limited thickness of the underlying rock in a way which is difficult to evaluate. Quantitative analyses by atomic absorption spectrometry (AAS) of bulk samples of the altered layer have been performed. The presence of elements such as Zn (320 ± 15 p.p.m.), Cu (156 ± 8 p.p.m.), V (25 ± 1 p.p.m.) and Pb (20 ± 2 p.p.m.), which are absent in the original rock was detected. These elements are evidence of the contribution made by atmospheric particulate. Mg is associated mainly with inorganic salts in coal fly-ash (1,000–10,000 p.p.m.), industrial dust (100–1,000 p.p.m.) and sea spray (3.7%)¹⁰. Particulate transport phenomena and deposition effects cause serious difficulties in determining an average Mg concentration in our system.

It has been thought^{4,11} that a high ratio of Mg/Ca favours the precipitation of dolomite, but this does not seem to be true in our case. However, the circulating solution is being analysed. Moreover the part played by pH, clay minerals and algae in the origin of dolomite must be considered. In none of the samples that we analysed, were dolomite and gypsum present at the same time. Because the hydration of SO_2 and its oxidation in sulphuric acid produces a lowering of pH in solution, we agree that the formation⁵ of dolomite is favoured by a high pH level.

The insoluble residues of some dolomite samples dissolved in 2% acetic acid show either no or only minor quantities of clay minerals. Thus no relationship between clay minerals and dolomite was detected which accords with the results obtained by Lumsden⁸. The presence of algae (*Gleocapsa* sp. and lichen soredium) and Basidiomycetes and Ascomycetes spores were observed.

Microclimatic conditions (temperature and humidity) led us to consider the possibility of the local reproduction of these microorganisms with consequent chemical alteration of the medium.

We have shown that authigenic dolomite is present in a different environment from those reported previously, at low (0–50 °C) temperatures at one atmosphere with a low Mg/Ca ratio and no clay minerals present. Furthermore the period of formation is fairly short. The upper limit is 500 yr and is even

lower if the effects of restoration carried out subsequently and the presence in the altered layer of elements like Pb, Zn, V, Cu, resulting from industrial development over the past few decades are considered. Dolomite crystals show dimensions up to 10 μm , which together with a low time period of formation suggests a high growth rate. A model for the formation of the altered layer could be: water vapour condenses on the surface and penetrates the porous altered layer. The less porous unaltered rock forming a discontinuity in the system favours an accumulation of water at the interface of the altered and unaltered rock. Here the transformation of the carbonates by the various elements dissolved in

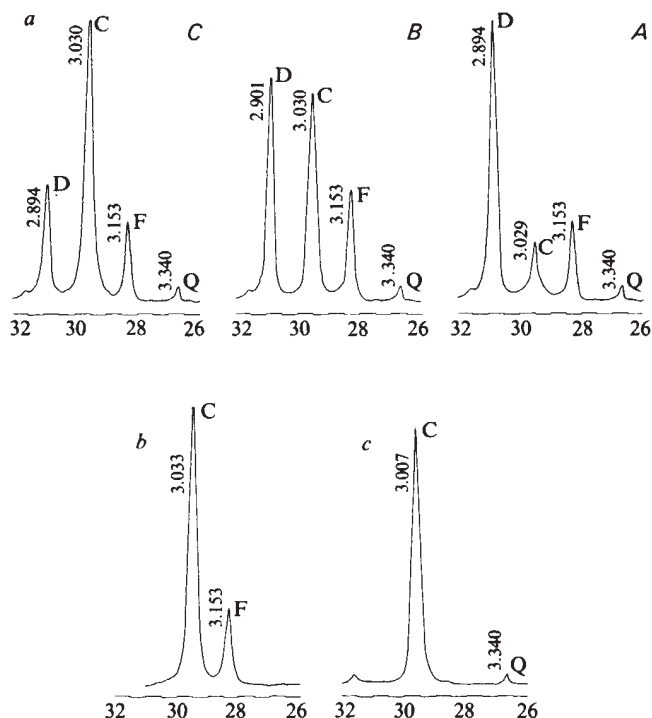


Fig. 3 X-ray Cu-K α diffraction patterns of: a, altered layer dolomite-calcite; b, unaltered marble; c, altered layer with only precipitation of calcite. The composition of dolomite (D) and calcite (C) was determined by the position of the d (104). Fluorite (F: d_{111}) and quartz (Q: d_{101}) were used as internal standards as their peaks fall reasonably close to the main calcite peak.

the water takes place. The growth of crust occurs in an inward direction so that the altered layers of more recent formation are in contact with the unaltered rock. As this phenomenon proceeds the formation of a protecting coating on the surface which reduce the chemical activity of the rock, or the detachment of the altered layer occurs.

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The Welsh 'geosyncline' of the Silurian was a fore-arc basin

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The Welsh 'geosyncline'¹, an example of the classic geosyncline, has often been viewed in terms of plate tectonics as a Japan Sea-type marginal sea²⁻¹⁵. It is generally agreed that in the Welsh area during Cambrian and Ordovician times, Anglesey, with its north-east and south-west extensions, was an island arc complex with a trench to the north-west, the trench being accompanied by a south-east dipping subduction zone which caused the Ordovician volcanism of Wales^{2,5,10-14}. Some authors believe that the Welsh region was the site of a back-arc tensional basin at the time of Ordovician volcanism. We believe that in Silurian times the subduction zone migrated landwards, that is southeastwards, due to tectonic erosion¹⁶, a process which has been implied for the modern Japan Trench¹⁷. Here, we compare the Welsh Silurian Basin with the modern Japan Sea and the sedimentary basins of the Pacific offshore of Japan, and conclude that a different interpretation is possible, that the Silurian Basin could have been a fore-arc basin.

In south-west Japan, between 132°E and 136°E in the vicinity of the Nankai Trough, the present subduction started at the earliest some 5 Myr ago¹⁸ and the subducting slab is thought to have now reached a depth of only about 50 km (ref. 19). Because the subduction is still young, there has been no Quaternary volcanism in this segment of the Japanese islands. We believe a similar situation existed in the Welsh region in the Silurian. In middle to late Llandovery times, the renewed subduction following a landward migration of the subduction

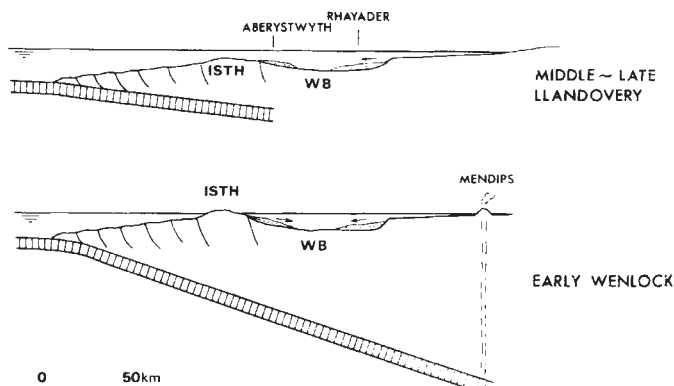


Fig. 1 Geotectonic profiles of the Welsh Basin in the Silurian. Arrows indicate derivation of clastics. WB, Welsh Basin; ISTH, Irish Sea Tectonic High.

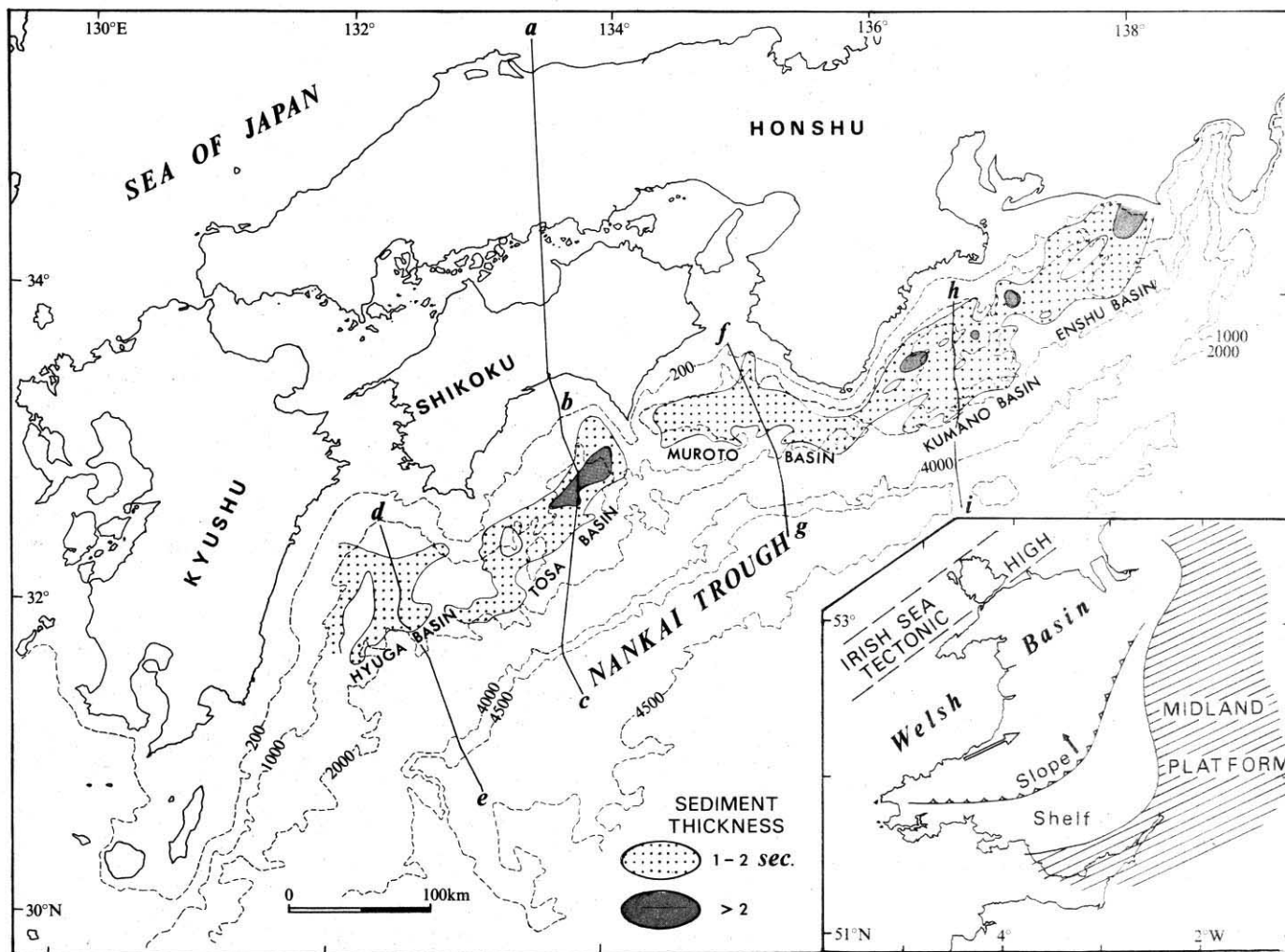
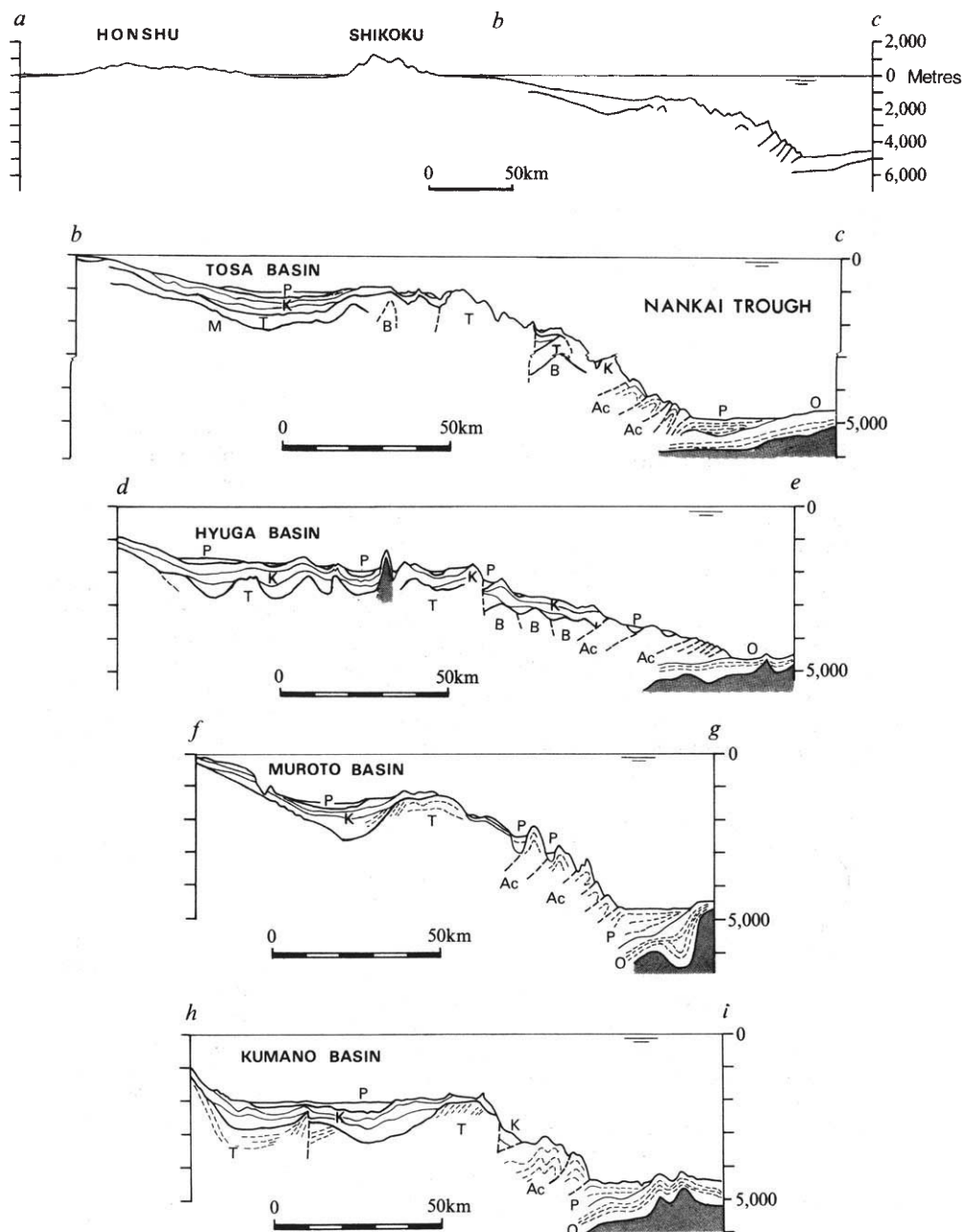


Fig. 2 Comparisons in dimensions between the Welsh Basin of Llandoveryan time (inset on same scale as main map) and the modern fore-arc basins behind the Nankai Trough off south-west Japan (modified from Okada *et al.*²²). White arrow in the Welsh Basin shows axial turbidity currents; black arrow shows lateral currents. *a-i* In the Nankai Trough area correspond to the profiles in Fig. 3.

Fig. 3 Geological profiles of the fore-arc basins behind the Nankai Trough off south-west Japan (modified from Okuda²⁵). M, B, acoustic basement; T, early-middle Miocene; K, late Miocene-late Pliocene; P, Pleistocene-Recent; Ac, accreted Pliocene to Recent; O, oceanic sediments. Shading indicates igneous rocks. *a-i* Correspond to areas shown in Fig. 2.



zone caused compressional uplift of North Wales and the adjacent Irish Sea Tectonic High. Such uplifts can be compared with the Oyashio ancient landmass postulated at the trench-slope break of the modern Japan Trench (Fig. 1)²⁰. At this stage the angle of the newly subducting slab was too small and the subducting edge of the slab too shallow to allow arc volcanism. Only by Wenlock times was volcanism again possible in Wales and adjacent areas (Fig. 1).

If the Welsh Basin of the Silurian could be represented as the Japan Sea-type marginal basin, then it should be characterized somewhere by tholeiitic ocean-floor basement and by ridge and trough features formed by rifting. No such characteristic features have yet been discovered. Conversely, the major faults found in Wales and the Welsh Borderland such as the Bala and Church Stretton Faults, are not dissimilar from those found in

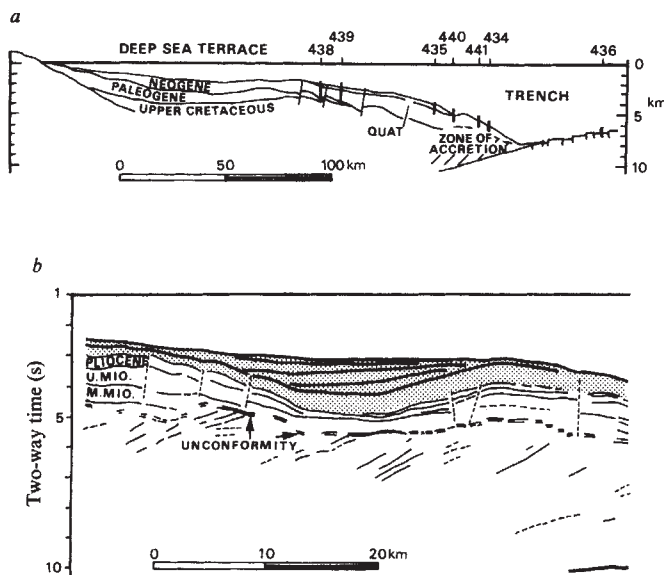


Fig. 4 Seismic profiles of the Japan Trench transect at 40°N *a*, General structural profile, redrawn from ref. 20. Numbers along the top of *a* refer to DSDP sites of Leg 56/57; indicated depth in km. *b*, Detailed seismic section of the fore-arc basin in *a* showing landward migration of depocentres¹⁷. U. Mio, Upper Miocene; M. Mio, Middle Miocene.

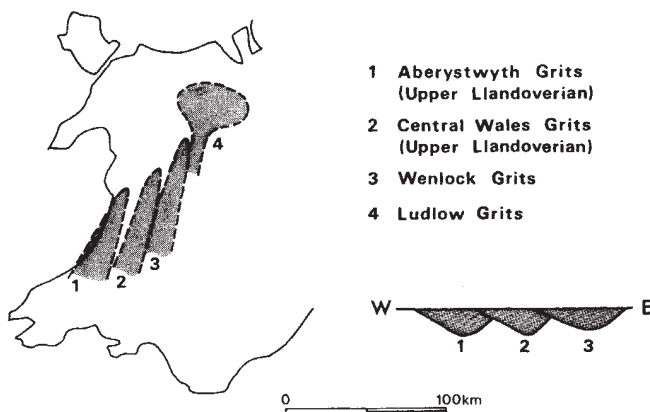


Fig. 5 Schematic diagrams showing landward migrations of depocentres of major Silurian turbidite sequences in Wales.

fore-arc basins—see, for example, those of the Sumatran continental margin²¹.

It seems possible to compare closely the Silurian Welsh Basin with modern arc-trench gap basins or fore-arc basins in several ways: tectonic position, volcanicity or its absence, the scale, size and shape of the basin and sedimentation types, styles and patterns. To emphasize this, we consider some fore-arc basins behind, that is landward, of the Nankai Trough (Figs 2, 3)²² and the Japan Trench. The Tosa Basin, for example, is about 80 km across and 200 km long and accommodates late Miocene to Quaternary sediments to more than 1 km in thickness. These sediments are characteristically terrigenous turbidites and moreover they exhibit the tendency of depocentres to migrate inwards or landwards with time as the trench-slope break tectonic high is uplifted (see Fig. 4, which illustrates the Japan Trench situation). A similar trend of migration of depocentres is seen in the succeeding stages of the Silurian in the Welsh geosyncline (Fig. 5).

Changes in the subduction angles inferred for the Welsh geosyncline can be explained by Kanamori's²³ cyclic evolutionary model of oceanic plate subduction. According to Niitsuma's estimates²⁴, the time length of unit cycles of plate subduction in the Japanese Neogene sequences is 10–20 Myr. Such an interval fits well with those of the Welsh Basin.

Such a multiplicity of coincidences between the fore-arc situation as has existed from early Neogene times on the Pacific side of Japan and the evidence from the Silurian rocks of Wales at least demands serious consideration of the conclusion that the Silurian Basin of Wales could have been a fore-arc basin.

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Age and magmatic evolution of Stromboli volcano from ^{230}Th – ^{238}U disequilibrium data

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The Eolian archipelago is one of the two active volcanic arcs in the Mediterranean. Of its seven islands, only Vulcano and Stromboli have erupted recently. Stromboli is well known for its continuous explosive activity. Whereas the relative ages of volcanism are fairly well known from stratigraphical methods^{1,2} there has been little absolute dating. Precise age determinations for the different islands are, however, necessary to study the evolution of the whole Eolian arc volcanism, notably the transition from calc-alkaline series to shoshonitic series^{3,4}. We report here the age determination of lavas on Stromboli using a method based on the radioactive disequilibrium $^{230}\text{Th}/^{238}\text{U}$ applied to volcanic rocks^{5–7}. The analytical techniques have been described elsewhere⁵. The activity ratio ($^{230}\text{Th}/^{232}\text{Th}$) is determined through α spectrometry and the U/Th ratio through isotopic dilution and mass spectrometry.

Stromboli Island can be divided into two parts, an older one to the east and a western, younger part to which the active craters of Stromboli belong (Fig. 1). The oldest part comprises three main formations⁸: (1) a basal pyroclastic complex with interstratified lava flows outcropping at the bottom of canyons and on the east coast (La Petrazza); (2) an intermediate complex made up of scoria and lava flows lying unconformably on the previous complex in the north-east; and (3) an upper complex or Vancori complex (lava flows and sills, tuffs in its uppermost part) which still forms the actual top of the volcano. The lavas of these old complexes belong to the calc-alkaline series and most are either K-rich andesites or Al-rich basalts^{4,8}. However, some rare shoshonites have also been observed⁹.

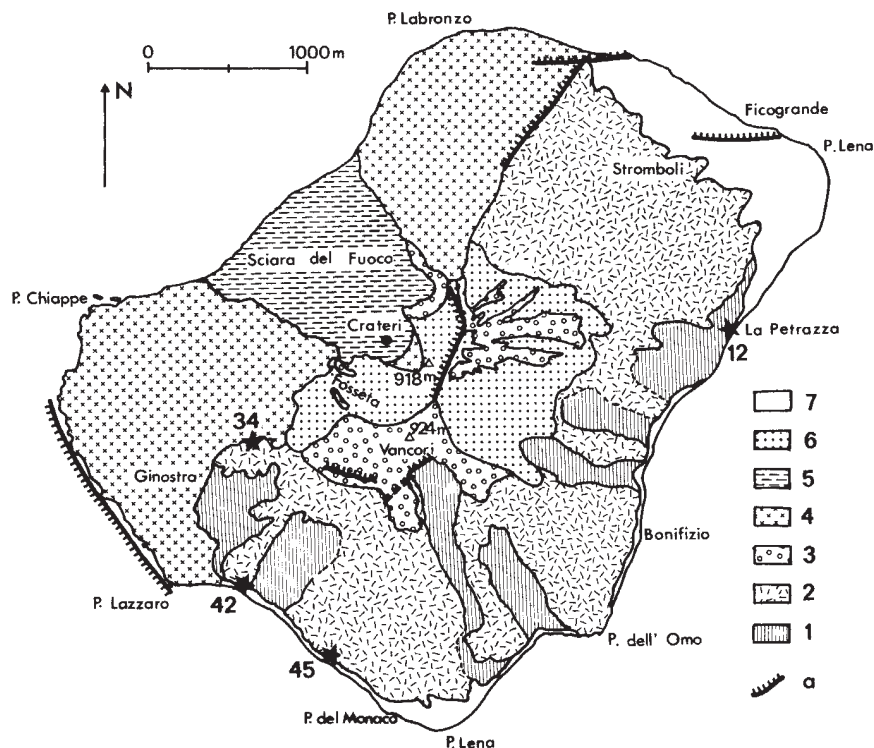
The younger, western part comprises several formations made of lava flows and scoria. The 'Sciara del Fuoco' consists of superimposed historical flows running all the way from the uppermost, active craters down to the sea. The lava from this young area are shoshonitic with a SiO_2 content usually in the range 50–53%, a variable but high K_2O content (up to 4% or more) and a $\text{K}_2\text{O}/\text{Na}_2\text{O}$ ratio of ~ 1 (refs 4, 10).

The samples analysed all come from the older eastern region of the island (Fig. 1). Specimen number 12 is from the oldest part of the basal complex around La Petrazza: the other specimens are from the intermediate complex on the south-west coast of the island (Stb 42, 45), and from the slope of the volcano itself (Stb 34; 400-m altitude). Samples Stb 12, 34 and 45 are K-rich andesites whereas Stb 42 is a Si-poor andesite according to Taylor's terminology¹¹ (F. Barberi, personal communication).

Analytical data and petrographical descriptions are given in Table 1: U and Th contents (Th from ~ 10 to ~ 20 p.p.m.) agree with other Th and U data^{12,13} on volcanics from the Stromboli calc-alkaline series. Our data confirm that these series have a higher content of hygromagmatophile elements than do the classic calc-alkaline series. On the ($^{230}\text{Th}/^{232}\text{Th}$), ($^{238}\text{U}/^{232}\text{Th}$) activity-ratio diagram (Fig. 2a) representative whole-rock values fall to the left of the equiline (first bisectrice) which indicates a ($^{230}\text{Th}/^{238}\text{U}$) disequilibrium ratio of > 1 .

In an activity-ratio diagram, representative data for minerals from a volcanic rock must fit an isochron of slope $(1 - e^{-\lambda t})$ thereby giving the age t of the rock. The intersection between the isochron and the equiline gives the initial ratio ($^{230}\text{Th}/^{238}\text{U}$)₀.

Fig. 1 Geological map of Stromboli (after ref. 8): 1, oldest complex (calc-alkaline series); 2, intermediate complex (calc-alkaline series); 3, Vancori complex (calc-alkaline series); 4, young shoshonitic volcanics; 5, Sciara del Fuoco; 6, present cinder cover; 7, alluvions and reworked volcanics; a, faults or ancient caldera edges.



The results of internal isochrone dating on samples Stb 12, 42 and 34 are plotted in Fig. 2. The most important mineral for this type of dating is titanomagnetite because of its higher Th/U fractionation relative to the whole rock (as we pointed out in our studies on lavas from Costa Rica and Etna^{5,6}). Nevertheless, for

Table 1 Analytical data and petrographical descriptions of samples from Stromboli volcano

Sample	U (p.p.m.)	Th (p.p.m.)	Th/U	$(^{238}\text{U}/^{232}\text{Th})\%$	$(^{230}\text{Th}/^{232}\text{Th})\%$
Stb 12*					
Whole rock	3.81	15.7	4.12	0.728	0.834 ± 0.016
Magnetite M1 80–20 μm	0.525	2.20	4.19	0.72	
Magnetite M2 20–8 μm	1.59	8.74	5.50	0.546	0.690 ± 0.012
Plagioclase Pl	0.377	1.41	3.73	0.802	0.883 ± 0.018
Clinopyroxene Cpx	0.316	1.49	4.73	0.634	0.767 ± 0.016
Stb 42†					
Whole rock	2.57	9.77	3.80	0.790	0.841 ± 0.008
Magnetite M1 80–20 μm	0.683	3.25	4.76	0.63	
Magnetite M2 20–8 μm	1.51	9.91	6.57	0.457	0.697 ± 0.007
Stb 34‡					
Whole rock	4.61	17.2	3.72	0.810	1.007 ± 0.017
Magnetite M1 80–20 μm	1.01	2.47	2.45	1.23	1.12 ± 0.02
Magnetite M2 20–8 μm	1.85	5.15	2.79	1.08	1.09 ± 0.02
Stb 45					
Whole rock	5.18	21.5	4.15	0.720	0.85 ± 0.01

* Stb 12: K-rich andesite with zoned plagioclase phenocrysts rich in glass inclusions, clinopyroxene, orthopyroxene and magnetite. The groundmass is made of plagioclase, pyroxene and magnetite microliths. Apatite is also present as needles in plagioclase.

† Stb 42: Si-poor, K-rich andesite with olivine, clinopyroxene and plagioclase (rich in glass and pyroxene inclusions) phenocrysts and magnetite micro-phenocrysts. The groundmass consists primarily of plagioclase and pyroxene microliths.

‡ Stb 34: K-rich andesite with zoned plagioclase phenocrysts rich in inclusions, clinopyroxene, orthopyroxene, biotite and magnetite in a groundmass of brown glass with tiny plagioclase and pyroxene microliths. Apatite is abundant as inclusions in plagioclase and pyroxenes.

§ Activity ratios: standard error on $(^{238}\text{U}/^{232}\text{Th})$ is estimated at 1%. The error on $(^{230}\text{Th}/^{232}\text{Th})$ is the α -counting error (1σ).

samples Stb 12 and 42, this fractionation occurs in the opposite direction, that is Th/U for magnetite is greater than for the total rock. For Stb 34 fractionation is 'normal'.

The age of Petrazza lava (Stb 12) from the eastern basal complex is $\sim 156,000$ yr ($^{+45,000}_{-32,000}$) and its initial ratio close to 1.16. The lack of precision on dating this lava is due to its great age and also, and mostly, to moderate Th/U fractionation between the different minerals and the whole rock. The age of sample Stb 42 from the intermediate complex on the southwestern coast is 61,500 yr ($\pm 6,500$) and its initial ratio $(^{230}\text{Th}/^{232}\text{Th})_0$ 0.880 (± 0.015). The age of sample Stb 34 from the upper part of the intermediate complex is 35,000 yr ($\pm 9,000$) and its initial ratio 1.084 (± 0.005).

These ages agree with the relative stratigraphical position of the different lava flows and can be used to infer a general pattern for the chronology of Stromboli volcanism: the oldest-emerged part of the island is at least 160,000 yr old, the formation of the intermediate complex probably started at least 60,000 yr ago and lasted until $\sim 30,000$ yr ago. The Vancori complex was then formed, followed by the younger western part of the island.

The transition to shoshonitic volcanism thus occurred $< 35,000$ yr ago. Barberi *et al.*⁴ and Keller³ consider that this significant transition corresponds to an important change in the subduction process underneath the Eolian arc. K_2O -rich shoshonitic lava could result from fast subduction of the lithosphere⁴ or from the presence of an isolated fragment of lithosphere sinking into the mantle³. The transition to shoshonitic volcanism must, then, be synchronous along to the strike of the subducting lithosphere. Seismic data show a 50 – 60° dip towards WNW (refs 14, 15), and Vulcano and Stromboli should thus be in a similar position relative to the subduction zone. Unfortunately, the age of early shoshonitic volcanism for Vulcano has not yet been exactly determined (though it is probably $< 100,000$ yr (ref. 3)). Our data put limitations on this model and will eventually allow age correlations with neighbouring islands of the Eolian archipelago.

There are too few petrographical and chemical data for older lava from Stromboli to allow a detailed study of magmatic evolution of this island; however, the ^{230}Th – ^{238}U disequilibrium method can be used to determine the main steps. Indeed, the study of the variation throughout time of the initial ratio $(^{230}\text{Th}/^{232}\text{Th})_0$ for lavas gives valuable information⁵.

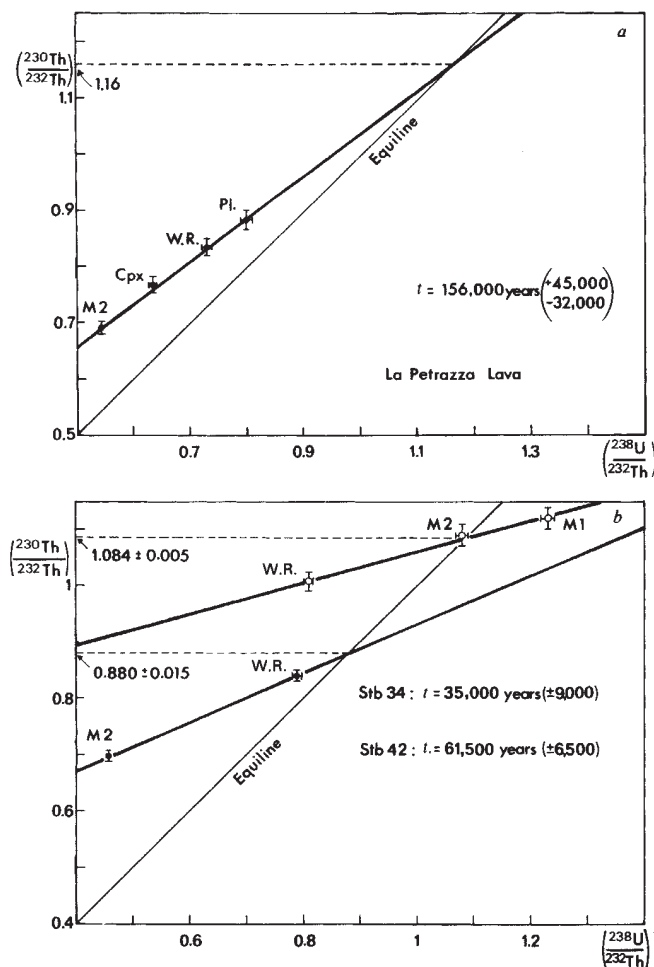


Fig. 2 Isochron diagram for: a, sample Stb 12; b, samples Stb 42 (●) and 34 (○).

When lavas derive from deep magma through evolution in a chemically closed system since a certain time T (the age of magma isolation), their initial ratios obey the following equation⁵:

$$(^{230}\text{Th}/^{232}\text{Th})_o = [(^{230}\text{Th}/^{232}\text{Th})_{Mo} - (^{238}\text{U}/^{232}\text{Th})_M]e^{-\lambda T} + (^{238}\text{U}/^{232}\text{Th})_M$$

where t is the age of lava and $(^{238}\text{U}/^{232}\text{Th})_M$ and $(^{230}\text{Th}/^{232}\text{Th})_{Mo}$ the initial ratios of the deep magma. This relation is valid as long as the $(^{238}\text{U}/^{232}\text{Th})_M$ ratio remains the same throughout fractional crystallization, which is probably the case for such hygromagmatophile elements except, perhaps, for the products resulting from late differentiation.

Indeed, Th/U ratios for calc-alkaline series seem to remain constant. Figure 3 shows Th and U values plotted for our samples as well as those of Guillard¹³ for calc-alkaline and shoshonitic series and those determined by Capaldi *et al.*^{16,17} for recent Stromboli lavas. On such a diagram, the two series can be readily differentiated on the basis of their Th/U ratios, ~4 for the calc-alkaline series and ~6.5 for the shoshonitic series, which indicates that the primary magma for the two series seems to be different.

Considering the hypothesis that the $(^{238}\text{U}/^{232}\text{Th})_M$ ratio of the deep primary magma has not been affected during fractional crystallization, one can use the above equation to study the evolution of initial ratios in a $(^{230}\text{Th}/^{232}\text{Th})_o - e^{-\lambda T}$ diagram. Representative data for lavas derived from the same deep magma evolving in a chemically closed system must fit a straight line of slope $[(^{230}\text{Th}/^{232}\text{Th})_{Mo} - (^{238}\text{U}/^{232}\text{Th})_M]e^{-\lambda T}$, and knowing the position of a lava on such a diagram and the $(^{238}\text{U}/^{232}\text{Th})_M$ ratio of the deep magma, we can then determine the evolution line for the initial ratios and find out whether other dated lavas belong to the same series—whether they derive from the same deep magma. This method has been used in Fig. 4: samples Stb 12 (156,000 yr) and 42 (61,500 yr) may belong to the same series whereas Stb 34 (35,000 yr) and present lavas from Stromboli seem to have a different origin. Thus, we have to distinguish at least three series:

- (1) A calc-alkaline series starting at least 160,000 yr BP and lasting until about 60,000 yr BP.
- (2) Another younger calc-alkaline series from 60,000 yr BP to <35,000 yr BP. The primary magma of this series seems to be identical with the previous series, the $(^{230}\text{Th}/^{232}\text{Th})_{Mo}$ ratios being very close.
- (3) The present shoshonitic series for which Th/U ratio is different from that of older shoshonites and would even be

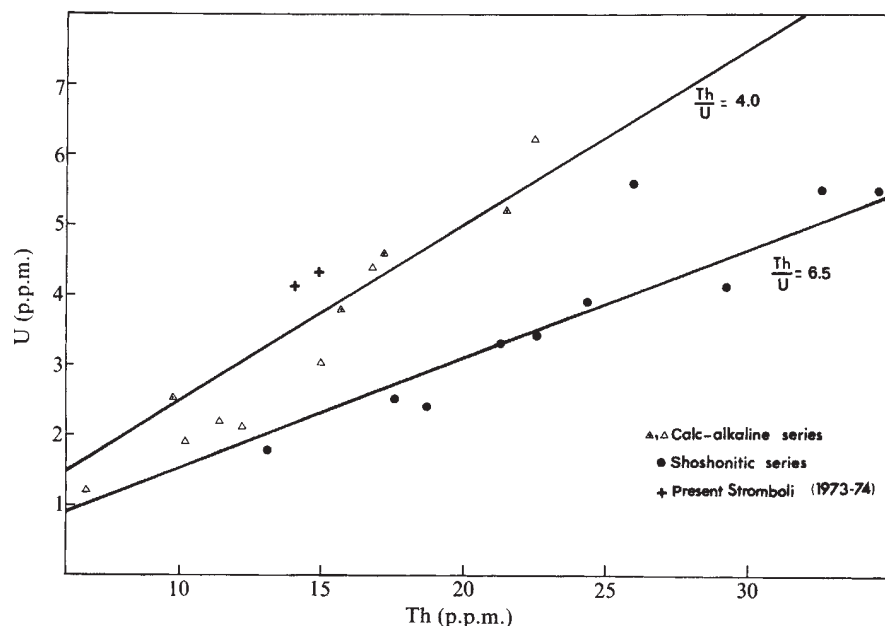


Fig. 3 Th/U diagram for Stromboli lavas: ▲, our data; △, ●, Guillard's data¹³; +, Capaldi *et al.*'s data^{16,17}.

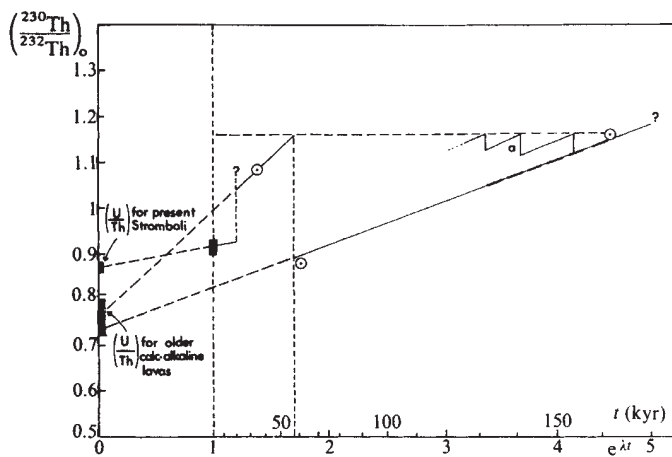


Fig. 4 $(^{230}\text{Th}/^{232}\text{Th})_0 - e^{-\lambda t}$ diagram (see text for explanation).

closer to that of calc-alkaline series (Fig. 3). This series would obviously be derived from an initial magma different from that of the calc-alkaline series as their $(^{230}\text{Th}/^{232}\text{Th})_{\text{Mo}}$ ratio is much lower (Fig. 4). Besides, the older shoshonites with high Th/U ratios could belong to an intermediate series not shown on Fig. 4.

Samples Stb 12 and 42 and sample Stb 34 which belong to two different series also have opposite Th/U fractionation trends for magnetite compared with the whole rock. This could be due to different crystallization conditions where oxygen fugacity could have an important role (in preparation). This parameter may explain differences between fractional crystallization of the calc-alkaline series and of the shoshonitic series^{4,18}.

The series defined above are based on the hypothesis of evolution in a chemically closed system of a deep magma successively feeding the dated eruptions. This deep magma can be isolated in a zone where it generated through partial melting, for example, at the top of the subducting lithospheric plate, or at an intermediate level.

The magma has probably not evolved in a superficial closed-system magma chamber for several tens of thousand years. If it had, then the initial ratio evolution for a given series would be correlated with the differentiation degree of lava, whereas there seem to be several cycles of differentiation, for calc-alkaline series, for example, slightly differentiated lavas alternating with more evolved ones⁸. Therefore, there may have been several reinjections of primary magma into the chamber but the basic magma added could not have the same $(^{230}\text{Th}/^{232}\text{Th})_0$ initial ratios would thus be much lower than that actually calculated for the oldest calc-alkaline series, for example. The evolution curve of $(^{230}\text{Th}/^{232}\text{Th})_0$ would be above the line corresponding to the evolution in a closed system (Fig. 4a). If $(^{230}\text{Th}/^{232}\text{Th})_0$ ratios variations actually correspond to the evolution line for a closed system, it implies a really deep magma evolving as a chemically closed system with a $(^{230}\text{Th}/^{232}\text{Th})_0$ ratio decreasing with time and imposing the general variation of the $(^{230}\text{Th}/^{232}\text{Th})_0$ ratios. The successive reinjection-differentiation cycles would thus be represented by straight segments on the evolution line representing the deep magma evolution (Fig. 4).

The proposed model would thus be: a deep permanent magma evolving as a closed system and feeding through successive reinjections one or several superficial magma chambers, where the main differentiation would take place through fractional crystallization. If this model were verified, there would be important geodynamic implications for the subduction beneath the Eolian arc and for the size of the deep magma reservoirs relative to subduction rates. The problem of calc-alkaline to shoshonitic volcanism transition cannot be solved here because of the lack of data especially on the older shoshonites. We have

shown that shoshonitic series seem to generate from an initial magma different from the calc-alkaline series primary magma which has a higher $(^{230}\text{Th}/^{232}\text{Th})_{\text{Mo}}$ ratio. Two hypotheses can be proposed for the origin of this magma: either it comes from a source region different from that of the calc-alkaline primary magma, with a different Th/U ratio, or it results from a modification of the calc-alkaline series primary magma through a more or less complex contamination process. A contamination by relatively high Th/U and low $(^{230}\text{Th}/^{232}\text{Th})_0$ must could explain the transition to ancient shoshonites. But there is no evidence for continental type crust underneath the Stromboli volcano, whereas such evidence does exist for other islands in the Eolian arc¹. Dupuy *et al.*¹⁹ use a fluid-transfer mechanism to explain the important enrichment of shoshonitic magma in hygromagmatophile elements such as K, U and Th. Detailed studies on ancient shoshonites might help solve the problem of their origin. In this respect Th/U ratio measurements and determination of initial $(^{230}\text{Th}/^{232}\text{Th})_0$ ratios would then be of great interest.

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Prehistoric shell assemblages from Franchthi Cave and evolution of the adjacent coastal zone

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Franchthi Cave, Greece, virtually continuously occupied from the late Palaeolithic to the Final Neolithic¹, is situated at the entrance to Koiládha Bay on the Gulf of Argos, where the sea meets a steep rocky shore just outside the cave entrance (Fig. 1). The deposits of the cave reveal much evidence of man's use of marine resources. For much of the occupational history of the cave, however, the shore was actually quite far away due to the glacial lowering of sea level for example, 6-7 km during the latest Palaeolithic. Perhaps this is why the earliest deposits examined contain little shell material. A second phase has a mixed shell assemblage of short duration (Fig. 2) but a third one from the late Mesolithic has abundant shell remains dominated by a single species. We show here that these shell assemblages reflect environmental changes resulting from the rising post-glacial sea level.

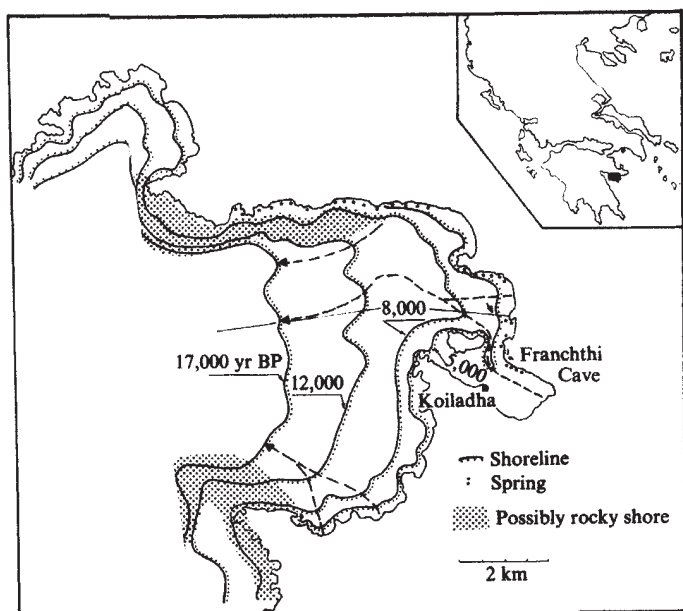


Fig. 1 Shoreline positions (yr BP) for the Franchthi area during the late Pleistocene and early Holocene. Based on contours prepared from British Admiralty chart 1518/1954. Data point spacing gives a contour position uncertainty of about ± 200 m. Sea-level rise curve uncertainty produces a shoreline position uncertainty of 200–500 m. Dashed arrows are streams. Solid lines are seismic reflection axes and arrow indicates a submerged bar after ref. 6.

From ~9,000 to 8,000 yr BP the inhabitants collected mainly *Cyclope neritea*², a small (~15 mm) species which lives in the littoral zone near the mud surface and also flourishes in brackish water. It is gregarious and a carnivore. Although small it is easy to catch because it occurs in large numbers in very shallow water³ and can be attracted with bait (M. Bishop, personal communication). It might be eaten raw or as a soup. At about 8,000 yr BP this assemblage is abruptly replaced by another, dominated by *Cerithium vulgatum*², a rock-dweller.

Although the ethnographical evidence provides instances of hunter-gatherers choosing to go long distances to collect delicacies⁴, this behaviour is the exception. Whatever the reason for the change in shell assemblages, we have adopted the view that a group will normally harvest a resource because it is accessible. We also assume that a change in economic or social behaviour may be triggered by environmental factors⁵. Obviously, both changes in shell composition could have been caused by various factors but here we discuss them in terms of an environmental/economic approach. Our interpretation rests on the fact that the post-glacial sea-level rise beginning 17,000 yr ago created a succession of coastal environments which must have influenced the lives of the inhabitants of Franchthi Cave.

There are two explanations for the dominance of *Cyclope* and its sudden replacement around 8,000 yr BP by the *Cerithium* assemblage (Fig. 2). First, *Cyclope* might have flourished on extensive, very shallow and sheltered nearshore flats near the cave. Such an environment might have existed temporarily during the early Holocene. Further migration of the shore into the bay and establishment of present conditions could have eliminated the species. Simultaneously, the advance of the sea produced rocky shores north of the cave, 2–3 km away, where *Cerithium* could be collected instead. Alternatively, the mouth of the river then flowing seaward between Franchthi and the island opposite⁶ might have been converted into a perhaps brackish estuary with quiet, shallow, sandy shores where *Cyclope* could be collected. Further rise of the sea destroyed this environment.

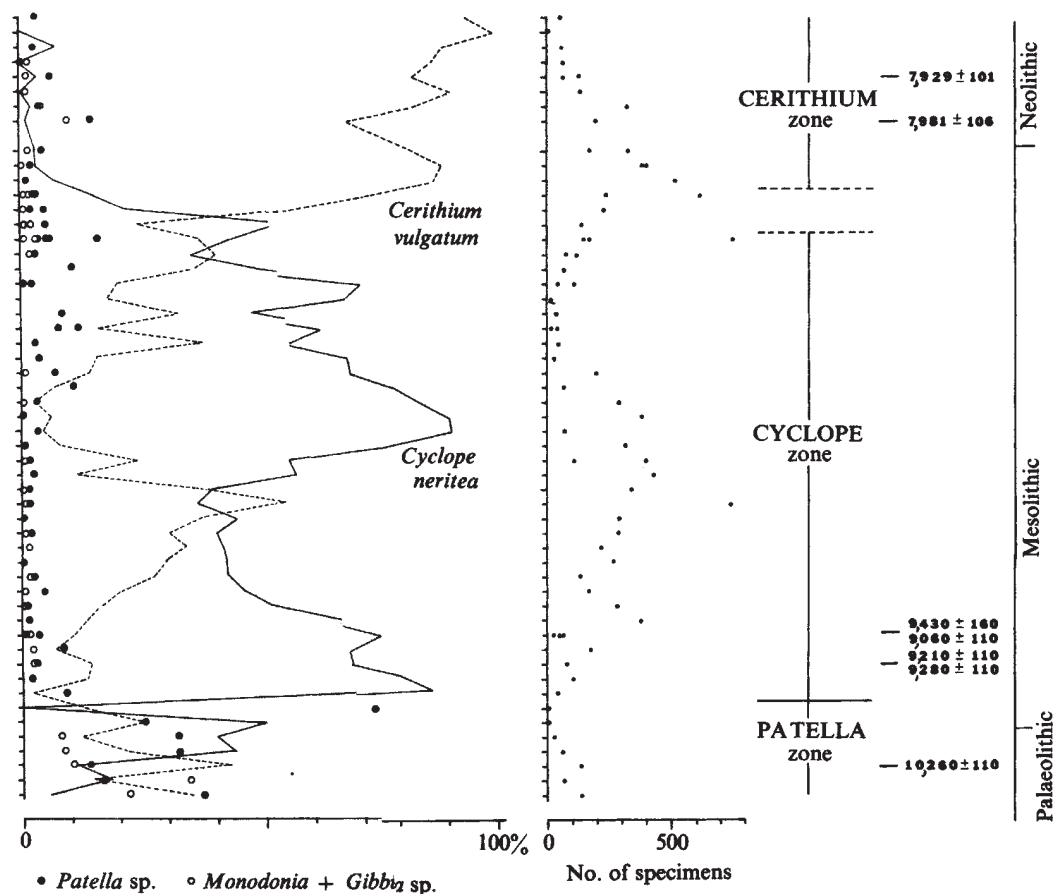


Fig. 2 Stratigraphy of late Palaeolithic through early Neolithic marine shell assemblages from a trench (F/AS) in Franchthi Cave. Sampled units are shown vertical to no scale. Left, percentages of most important species; centre, total number of specimens in each sampled unit; right, shell zones and radiocarbon dates (yr BP).

Figure 1 shows a series of past shorelines using the shelf bathymetry and sea-level positions determined from a post-glacial sea-level rise curve. The present best value for the maximum lowering is 90–95 m and the shape of the following rise curve is still uncertain^{7–10} and complicated by isostatic responses to ice removal and water addition^{11,12}. Comparison of various proposed curves shows vertical uncertainties of 5–15 m for each of the chosen intervals which, for the shelf topography in our area, translate into horizontal uncertainties of a few hundred metres. For our purpose the shorelines so determined are adequate. Little modern sediment is present on the shelf⁶ and the present water depths are a sufficient guide. In Koiládha Bay, where sediments are thicker, we have used coastal outlines from a more detailed study⁶.

It has been argued that the Holocene sea-level rise around the Peloponnese was accompanied by vertical crustal movements^{13–16}, including a subsidence of 1–2 m kyr⁻¹ for the southern Argolid. If true this would shift all shoreline positions seaward by 0.51 km. The evidence for the southern Argolid is weak^{13–17}, but if true it lengthens the walking distance to the sea before 8,000 yr BP by at least 1 km.

Three aspects of the reconstructed early Holocene shores are of interest; the rocky or sandy/muddy nature, the distance to the cave, and the presence of sheltered shoals or brackish lagoons or estuaries. The present coast is mainly rocky with offshore rocky flats and occasional pocket beaches. Large sand or gravel beaches exist only in Koiládha Bay and the bay north of Franchthi. During the early Holocene the situation was quite different. The smooth offshore topography with a slope of less than 0.6° implies an alluvial coastal plain, confirmed by seismic reflection data⁶. Only along the extreme north and south coasts a rough bottom suggests a possible rocky shore before 8,000 yr BP. Thus only 30–45% of the coast was rocky as contrasted to >75% today.

During the late Palaeolithic and much of the Mesolithic the coast was from 3.5 to 6.5 km away depending on whether one assumes subsidence. It is thus not surprising that whatever the shore may have yielded in seafood did not find its way into the cave deposits in quantity. The gradual approach of the sea to 4 km is, however, reflected in the appearance of some shells around 11,000 yr BP.

Shortly before 9,000 yr BP shells increase in number (Fig. 2). At this time the simple, straight shoreline had begun to develop embayments and capes ~2 km away, but remained sedimentary. Of the several streams the largest, the Koiládha river, was much larger than its modern successor⁶ and flowed semi-permanently until ~7,000 yr BP. On the open coastal plain its valley was wide and had little effect on the shoreline, but as the sea approached Franchthi a bay was formed around 9,000 yr BP in the here much steeper and narrower valley. By 8,000 yr BP a narrow estuary existed just west of the present cave entrance which may have been closed by a bar at the outer shore; a sediment ridge occurs at the appropriate spot and depth (25 m) on seismic profiles⁶. Given the larger and more permanent river flow and the presence of copious freshwater springs at the foot of the Franchthi cliffs¹⁸ it is possible that the estuary was brackish or had a variable salinity rather than the high salinity typical for lagoons in the Argolid today. Such an estuary, with sheltered and shallow shores, would provide an ideal habitat for *Cyclope* within a few hundred metres of the cave.

Thus the environments postulated earlier are probable at the right time and in the right place to explain extensive utilization of *Cyclope* on an open shore or in a sheltered and perhaps brackish bay¹⁹. Later, between 8,000 and 7,000 yr BP, further advance of the sea eliminated the bar⁹ and opened the estuary. Simultaneously, however, the limestone cliffs north of Franchthi became part of the littoral zone and their rocky shores provided a good habitat for *Cerithium* within a few kilometres from the cave.

Bones of large tunny appear at the base of the *Cyclope* zone, a species hitherto not used¹⁸. N. J. Shackleton (personal communication) has postulated an increase in biological productivity

in the Aegean at this time which might explain this use of a new species. We also suggest that the development of an embayed coast around 9,000 yr BP may have facilitated the capture from shore of this open sea fish. The same productivity increase would also have enlarged the shellfish available.

During the next few years much evidence from Franchthi will become available to test and amplify ideas such as those we have presented. We hope that this paper will stimulate the integration of archaeological and environmental information.

In the summer of 1980, we found abundant live *Cyclope neritea* near the beach at the head of the Gulf of Argos between Nauplion and Nea Kios, and also in small numbers in the Bay of Astakos in Acharnania. In both cases *Cyclope* lived at the surface of fine silty-muddy sand in very shallow depths of 5–15 cm, on quiet and extensive shallow flats off the beach, an environment that corresponded exactly to the first of the two postulated here.

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Radiometric dating of sediments using fission tracks in conodonts

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Conodonts are microfossils which are commonly found in marine rocks of Cambrian to Triassic age. Although their biological affinities are difficult to assess, conodonts are valuable stratigraphical indices for much of their geological range¹. Recent work has also established that conodont colour alteration indices (CAI) are useful guides to diagenetic temperatures and hence burial depth². Fission tracks³ in conodonts allow measurement of uranium concentrations and estimates of 'age' to be made using isotopic methods⁴. We report here that fission tracks counted in irradiated, thermally unaltered (as indicated by CAI) middle Palaeozoic conodonts indicate typical uranium concentrations of ~1 part in 10⁹, with some samples higher. A single specimen of *Siphonodella* from the Lower Mississippian yielded an age estimate of 380 ± 140 Myr consistent with conventional interpolations. This method may also allow the unroofing of deeply buried sediments to be dated.

Table 1 Summary of data

Sample code	Mineral	Spontaneous		Induced		Fluence (ϕt) neutrons cm ⁻²	T (Myr)	$\pm 1\sigma$ (Myr)	No. of grains or fields	p.p.b.
		Track cm ⁻²	Tracks†	Tracks cm ⁻²	Tracks†					
C-2-22*	Apatite	—	—	2.0×10^3	5	3.0×10^{15}	—	—	8	1.9
C-2-22*	Apatite	—	—	2.6×10^3	4	3.0×10^{15}	—	—	6	2.4
C-2-32	Apatite	—	—	8.1×10^4	101	1.62×10^{17}	—	—	6	1.4
C-2-33	Apatite	—	—	2.4×10^4	52	1.62×10^{17}	—	—	7	0.4
CDR-2	Apatite	—	12	—	—	2.0×10^{16}	—	—	—	—
CDR-4	Apatite	—	10	—	35	2.0×10^{16}	380	± 140	—	—

* Slide contains two conodonts.

† No. of tracks actually counted to determine the reported track density.

Conodonts consist of carbonate apatite and trace amounts of organic material⁵ and their structure consists of concentric lamellae, usually around a single growth centre. Lamellae of many conodonts average 2–3 μm thickness, with some interlamellar spaces of 0.5–1.0 μm thickness⁶. Individual crystallites are generally smaller than 0.01 μm and optically unresolvable (R. Phillips, cited in ref. 7). The crystallites are predominantly co-parallel, with optic *c*-axes parallel to the direction of growth (perpendicular to lamellae) in most cases^{8,9}.

The heavy nuclear particles released from spontaneous fission of ²³⁸U leave narrow damage tracks in most dielectric solids, generally 8–20 μm long (track lengths vary among mineral species). In crystals, the trajectory is marked by vacant lattice sites and/or interstitial atoms. These features are permanent unless the solid is heated to its annealing temperature, at which point the tracks begin to disappear³. In general, fission tracks are exposed by chemically etching random polished surfaces of solids, and examining the specimen optically at high magnification. Etch kinetic arguments, based on the expectation of high dissolution rates along crystallite boundaries, have discouraged earlier efforts to find fission tracks in conodonts.

Assuming reasonable etch efficiency, the feasibility of fission track work depends on the weight concentration of uranium (*c*) and the age (*A*) of the sample. Fleisher and Price³ suggested a minimum track density of 100 cm⁻² for routine searches, corresponding to $cA \geq 0.3$. This requires ~2 h in favourable conditions. For middle Palaeozoic conodonts (300 Myr), this corresponds to $[U] \geq 1$ part in 10⁹. Because conodonts are small (~1 mm), $cA = 0.3$ will substantially underestimate the time required to count many grains instead of a single homogeneous surface.

For dating, only the relative densities of spontaneous and induced tracks in the same specimen are needed. In contrast, using fission tracks to measure uranium concentration requires determining the specimen size, the number of spontaneous (fossil) tracks (or, alternatively, annealing the sample to remove pre-existing tracks), and counting the tracks from fission events caused by irradiation with a known dose of thermal neutrons. In this context, the first step is to establish methods which allow the tracks to be seen and which allow the conodonts to be handled for irradiation.

Surface preparation requires mounting and fine polishing. Each set of conodonts was randomly mounted in epoxy wafers and one surface ground until all specimens were exposed. The mount was then reversed, ground face to glass, and cemented to standard petrographical slides. Samples were ground to ~0.05 mm and the surface finally polished with 0.25 μm diamond grit—the minimum surface roughness acceptable for high magnification optical searches. Parallel-ground specimen surfaces seem crucial for reliable optical examination. After many trials, we chose as standard etch 0.25% nitric acid for 60 s (ref. 4).

Following a thorough search (using transmitted light differential interference contrast microscopy at $\times 1,250$) to determine the number of spontaneous tracks, the wafer was cleaned and removed from the glass slide for irradiation (both glass and Lexan became unacceptably radioactive when irradiated).

Each sample was wrapped in aluminium foil, as was the NBS calibration glass¹⁰, when used.

A thermal neutron dose of 2.0×10^{16} neutrons cm⁻² yielded usable results (Brookhaven National Laboratory, HFBR facility, V-II Port, D. Rohrer, personal communication). Epoxy-embedded, foil-wrapped samples were 'cool' enough for shipment almost immediately, and handling required only modest precautions. No special facilities were required.

Irradiated wafers were remounted on petrographical slides (for ease of handling), reground and etched again for optical examination and track counting. Grinding to a new surface maintained a 'solid' counting geometry—the expectation of finding track fragments from the solid volume instead of a half-space.

We obtained the following results: (1) Middle Ordovician simple conodonts and conodonts with 'white-matter'¹ which we tested darkened and became opaque during irradiation; we were unable to count tracks. (2) Although the track densities were very low, the distribution of induced tracks in platform conodonts appeared uniform, with no apparent concentration near edges, interlamellar spaces or other obvious features. We inferred that the uranium was disseminated in the apatite structure. Altschuler *et al.*¹¹ have suggested that uranium in conodonts would replace calcium. This is reasonable because the ionic radii of divalent Ca (0.99 Å) and tetravalent U (0.97 Å) are so similar¹². (3) Our best results were obtained with platform conodonts which showed no visible colour alteration (CAI = 1). Induced track densities of 2.0 – 2.6×10^3 tracks cm⁻² and 2.4 – 8.1×10^4 tracks cm⁻² for 3×10^{15} and 1.6×10^{17} thermal neutrons cm⁻², respectively, yielded concentrations of 1.9–2.4 and 0.4–1.4 parts per 10⁹ (p.p.b.) (Table 1), comparable with estimates by Naeser (personal communication). In eight nonirradiated specimens searched for fossil tracks, we found no tracks in five specimens, one each in two, and two tracks in one specimen. These track densities are expected for Palaeozoic samples with ~1 p.p.b. [U]. (4) Two specimens tested 'blind' (CDR-2 and CDR-4) yielded 12 and 10 well defined fossil tracks respectively, indicating order-of-magnitude higher [U] than other samples studied. CDR-2 was ground off inadvertently while repolishing after irradiation, but CDR-4 yielded 35 tracks from which we compute an age of 380 ± 140 Myr, from $A = 6.45 \ln [1 + 0.76 \Phi t (N_s/N_i)]$, where age is 10⁹ yr, Φt is the thermal neutron dose in 10¹⁷ neutrons cm⁻² and N_s/N_i is the density of spontaneous (induced) fission tracks in the solid. In addition to the 10 (spontaneous) and 35 (induced) unambiguous tracks (see ref. 3), there were respectively 6 and 12 'track-like' markings rejected during counting by M.D. In addition to the criteria of ref. 3, we compared the observed distribution of track lengths and orientations with that expected for randomly oriented tracks. The two specimens counted were large platform conodonts of the genus *Siphonodella* from the lower Chappel Limestone, Llano region, Texas, locality C-25 of ref. 13. The age of this interval is given as 340 Myr (ref. 14).

Thus, it is possible to count fission tracks in conodonts and to compute reasonable age estimates from these data. Many improvements in procedures could be made. Our preparation routines work well but are tedious: for example, methods which

allow irradiation of specimens with their mounts would save time and eliminate the risks inherent in handling unsupported epoxy wafers. Fossil track counting statistics would be greatly improved by examining more than one surface on each specimen: multiple grind-etch-count cycles will greatly increase the number of tracks per conodont without affecting the induced track densities. We have limited information on the distribution of uranium concentrations in conodonts, but over 90% of the conodonts we have tested have had very low [U] (~1 p.p.b.). The precision of age estimates improves with the square root of the number of tracks counted. A reasonable target would be 100 spontaneous (fossil) tracks/age determination, which implies 10–100 conodonts examined for each accurate age determination.

Fission track dating of conodonts at stratotype boundaries and elsewhere may challenge conventional indirect age estimates based on correlation and interpolation from igneous rocks¹⁵. However, the scarcity of fission tracks in our conodonts suggests that much effort will be required to rival the precision of good radiometric determinations published for igneous rocks.

Nonetheless, the general dichotomy between (isotopically) datable but fossil-free igneous rocks and fossiliferous sedimentary rocks (which are the basis of the relative geological time scale) has complicated stratigraphical codes¹⁶. Except for ¹⁴C dating of uppermost Pleistocene fossils, direct dating of fossils has been virtually impossible, but by eliminating the need to correlate datable rocks with those whose position in the stratigraphic column is secure errors may be reduced. The benefits of directly dating mid-Palaeozoic rocks justify studying fission tracks in conodonts.

The greatest value of dating conodonts directly probably lies in studying the thermal history of sedimentary basins¹⁷. Because the annealing temperature of apatite is ~90–100 °C, corresponding to burial of 3–5 km (ref. 18), the fission track 'clock' only begins to run as deeply buried sediments are uplifted and unroofed. If we can count tracks in thermally altered conodonts, we can date the time at which the overburden thinned (structurally or by erosion) to 3–5 km. (If one tests conodonts from various burial depths in the same section, the fission track 'ages' (unroofing times) will decrease with increasing depth (depositional age) in the rock column, as seen in igneous and metamorphic rocks in an Alaskan drill hole¹⁹.)

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First pterosaur from Australia

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We report here the first evidence of pterosaurs from Australia. Our report is based on fragmentary material from marine Lower Cretaceous sediments of the Eromanga Basin, western Queensland. These fragments represent at least one pterosaur allied to the well-known *Pteranodon*^{1,2}, and establish a new southern limit of distribution for Cretaceous flying reptiles.

The material (Fig. 1) comprises three uncrushed specimens—the front of the fused mandibles (QM F10613), a single vertebra (QM F10614) and an isolated left scapulocoracoid (QM F10612). These were collected from flaggy limestones of the Toolebuc Formation at an unnamed locality 13 km south of Hamilton Hotel and about 70 km east of Boulia, west Queensland. The skull fragment and vertebra came from a single slab, and the scapulocoracoid was found about 500 m away. The Toolebuc Formation consists of crystalline and, in places, concretionary limestones with minor bands of shale; it has been dated as Lower Cretaceous (Albian)^{3,4} on the basis of its rich and varied marine fauna (including lamellibranchs, gastropods, belemnites, ammonites, fishes and marine reptiles).

The skull fragment (Fig. 1a, b) comprises the slender extremities of both mandibles, showing several characteristic pterosaur features: (1) the long fused symphysis; (2) the interior

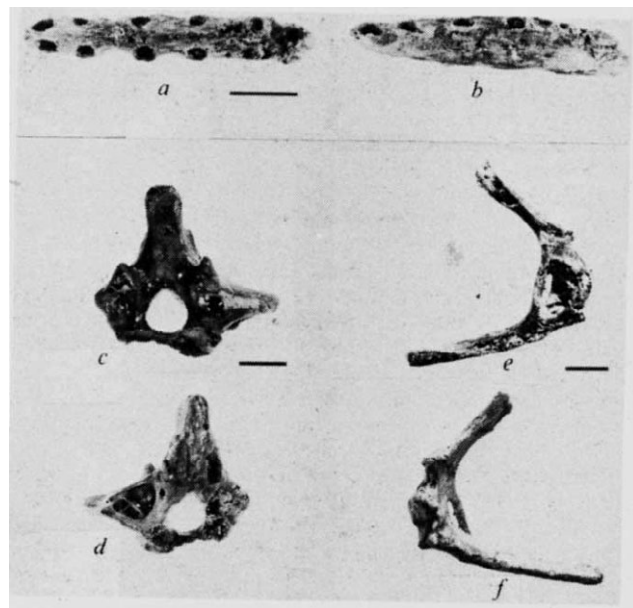


Fig. 1 a, Anterior region of mandibles of pteranodontid (Queensland Museum (QM) F10613) from Toolebuc Formation of Queensland, in palatal view. Note replacement crown in fourth left alveolus. Scale bar, 2 cm. b, The same in lateral view. c, Vertebra of pterosaur (QM F10614) from the same locality, in anterior view. Scale bar, 1 cm. d, The same in posterior view; note large aperture into hollow transverse process. e, Pteranodontid scapulocoracoid (QM F10612) in anterior view. Scale bar, 2 cm. f, The same in posterior view.

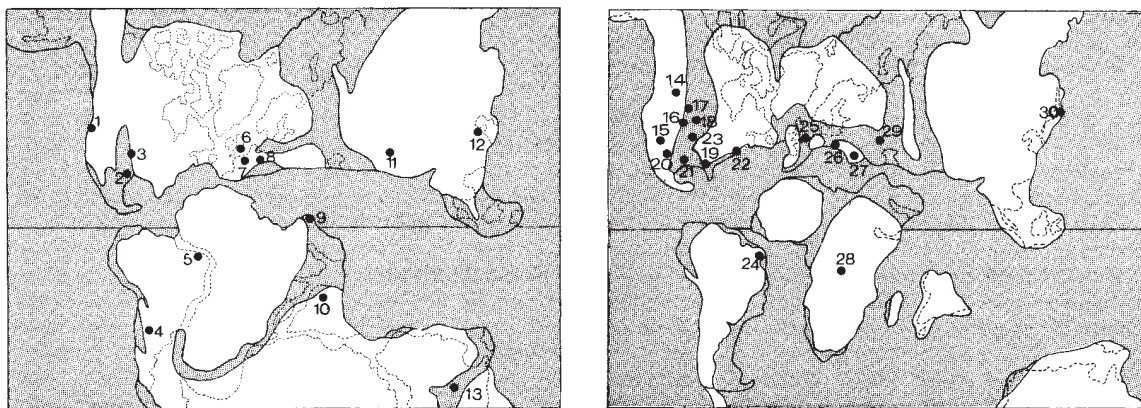


Fig. 2 Distribution of Cretaceous pterosaurs. Lower Cretaceous occurrences (left): 1, Oregon, *Pteranodon*?; 2, east Texas, unidentified; 3, Kansas, *Apatomerus*; 4, San Luis, *Pterodaustro* and *Puntanipterus*; 5, Ceara, *Araripidactylus* and *Araripesaurus*; 6, southern England, *Criorhynchus*, *Ornithocheirus* and *Ornithodesmus*; 7, France, *Ornithocheirus*; 8, Germany, '*Ornithocheirus*'; 9, Jordan, *Titanopteryx*; 10, Rajasthan, unidentified; 11, Dzungaria, *Dsungaripterus* and *Noripterus*; 12, Shandong, unidentified; 13, Queensland, aff. *Ornithocheirus*. Upper Cretaceous occurrences (right): 14, Alberta, 15, Arizona, 16, Wyoming, 17, Montana, 18, South Dakota and 19, Alabama, unidentified; 20, west Texas, *Quetzalcoatlus*; 21, east Texas and 22, Delaware, *Pteranodon*; 23, Kansas, *Nyctosaurus* and *Pteranodon*; 24, Paraíba, *Nyctosaurus*; 25, southern England, *Criorhynchus*, *Ornithocheirus* and *Ornithostoma*; 26, Austria-Bohemia, '*Ornithocheirus*'; 27, Transylvania, unidentified; 28, Zaire, cf. *Ornithocheirus*; 29, Petrovsk, *Ornithostoma*; 30, Hokkaido, *Pteranodon*.

of the jaws, exposed through breaks, was coarsely cancellous, or perhaps hollow; (3) the flat lateral surface of each ramus slopes down and inwards, giving a triangular cross-section (slightly deeper than wide); (4) the oral surface is transversely convex with a narrow median groove marking the line of the symphysis; (5) a distinctive arrangement of the teeth.

On each side are five deep and widely spaced alveoli, whose thin outer walls form distinct bulges in the jaw margins. Each alveolus is elliptical (with long axis longitudinal), and opens upwards and slightly laterally. The first alveolus on each side evidently carried a near-procumbent tooth; the second is more steeply inclined, whereas the following alveoli are almost vertical. Structure and size of the alveoli indicate a thecodont, near-isodont dentition at the front of the lower jaw. The fourth left alveolus contains a damaged replacement tooth; its crown is latero-medially compressed, and seems to have been tall and sharply pointed. In all respects the specimen is very similar to jaw fragments attributed to *Ornithocheirus*^{5,6} from the Cambridge Greensand (Cenomanian^{7,8}) of England.

The single vertebra (Fig. 1c, d) is incomplete, but shows most of the neural arch and the anterior part of the centrum. The centrum is relatively small and has a deeply concave anterior face, kidney-shaped in outline (concave above) and much broader than high. The walls and processes of the arch are virtually hollow, containing large cavities traversed by fine struts of bone. This system of cavities opens to the exterior in three places (seen on the well-preserved right side of the specimen): through a large triangular opening in the rear surface of the stout transverse process, and through smaller openings beneath the transverse process and below the base of the prezygapophysis. The robust neural spine is almost square in profile; its anterior margin is thick and flat, whereas its rear margin is narrower and carries a shallow vertical groove. The stout prezygapophyses have large articular facets inclined at about 30° from vertical; the postzygapophyses are smaller and sharper, each supported by a low ridge extending up and back from the base of the transverse process.

This pneumatic vertebra, with its depressed and probably procoelous centrum, is clearly from the presacral region of a pterosaur^{6,9}. The postzygapophysial facets are much smaller than the prezygapophysial, which may indicate that the vertebra was transitional between the flexible cervical series and the rigid notarium^{6,9}. The grooved rear margin of the neural spine suggests an articulation with the front of the notarium. Detailed comparisons are difficult because the vertebrae of other pterosaurs are usually crushed flat. Presacral vertebrae of *Pteranodon*

have similar oblique ridges supporting the postzygapophyses, but have relatively thin neural spines and seem to lack pneumatic openings¹. The rather thick neural spine is matched in *Ornithodesmus latidens*¹⁰, from the Wealden of England, and in the notarium of *Dsungaripterus weii*¹¹, from the Lower Cretaceous of China.

The scapulocoracoid (Fig. 1e, f) lacks only its uppermost tip and parts of the antero-medial margin. In postero-lateral view the specimen is roughly V-shaped (open posteriorly), with scapular and coracoidal wings enclosing an angle of about 75°. It shows a combination of features unknown in vertebrates other than pterosaurs^{6,9,12}: (1) A cancellous interior overlain by a smooth cortex less than 1-mm thick, revealed at the damaged areas. (2) Scapula and coracoid are basically rod shaped with oval cross-sections, expanding considerably where they meet to form the glenoid; the intervening suture has been obliterated by fusion, but probably ran horizontally through the central and deepest part of the glenoid. (3) On the medial side the glenoid region is strengthened by a vertical buttress enclosing a narrow slit-like fenestra. (4) The large glenoid opens laterally, and slightly to the front, with margins developed into thick 'lips' of bone. It is essentially an open groove, aligned antero-posteriorly, with flattened upper and lower walls meeting at slightly more than a right angle. In outline it resembles three-quarters of a square: its upper (scapular) wall is about twice as long as its lower (coracoidal) wall, and forms a distinct overhang posteriorly. (5) The scapula is nearly straight, and extends medially as well as up and backwards from the glenoid; this oblique, and partly transverse, orientation suggests that the scapula may have articulated with a notarium^{9,12,13}.

Just above the postero-dorsal corner of the glenoid the rear margin bears a prominent triceps tubercle¹². The coracoid is slightly curved (convex downwards), and about halfway along its dorsal surface bears a low longitudinal crest probably marking the coracobrachialis attachment². The tip of the coracoid is damaged, but enough remains to show a rounded surface for articulation with the sternum.

In general appearance the specimen is very similar to scapulocoracoids of the Upper Cretaceous pterosaurs *Nyctosaurus*¹⁴ and *Pteranodon*¹, the most obvious difference being the less strongly developed triceps tubercle in these latter forms. The specimen seems to be virtually identical to an incomplete scapulocoracoid attributed to *Ornithocheirus sedgwickii*^{5,6}, from the Cambridge Greensand of England.

Evidently, the Toolebuc material represents one or more pterosaurs of the suborder Pterodactyloidea. The specimens are

most closely matched in pterodactyloids such as *Ornithocheirus* and *Pteranodon*, and the medial buttress of the scapulocoracoid occurs only in certain pterodactyloids¹². In addition, there is indirect evidence of a notarium, a structure unknown outside the Pterodactyloidea. It is difficult to assign the Toolebuc material to a family because the two most similar forms, *Ornithocheirus* and *Pteranodon*, are at present in different families¹². The Toolebuc scapulocoracoid is nearly identical to that attributed to *O. sedgwickii*^{6,9}, but this species is known only from dissociated fragments and the referred scapulocoracoid might belong to another Cambridge Greensand pterosaur, *Ornithostoma seeleyi*¹⁵. *Ornithostoma* may, in turn, be identical with *Pteranodon*^{2,6,15}. The jaw fragment is clearly from a pterosaur unlike the toothless *Pteranodon*, and is most closely matched in *Ornithocheirus*. Adherence to the pterosaur classification given by Wellnhofer¹² would require assignment of the Toolebuc jaw fragment to one family (Ornithocheiridae) and of the scapulocoracoid to another (Pteranodontidae). Distinctions between Ornithocheiridae and Pteranodontidae are, perhaps, of doubtful validity, largely because material attributed in the past to *Ornithocheirus* is fragmentary and of uncertain status. The problem is exacerbated by a possibility that the Toolebuc material represents more than one animal. In view of these difficulties, we recommend that the Toolebuc material be provisionally assigned to the family Pteranodontidae *sensu lato* (including Ornithocheiridae *sensu stricto*), following the usage of Romer^{6,16}. The Toolebuc pterosaur material may be identified as aff. *Ornithocheirus*.

The best-known members of the Pteranodontidae *s.l.*, *Nyctosaurus* and *Pteranodon*, are of Upper Cretaceous age, whereas the earliest representatives come from about the boundary between Lower and Upper Cretaceous: from the Cambridge Greensand (Cenomanian^{7,8}) of England^{6,12,15} and the Hudspeeth Formation (Albian, possibly Cenomanian¹⁷) of Oregon¹². The Toolebuc pterosaur is certainly Albian in age^{3,4}, and hence, one of the earliest pteranodontids yet discovered.

Pteranodon and its allies were probably soaring maritime creatures², similar in their habits to oceanic birds of today, and it is not surprising that they should have been widely distributed. Even so, few pteranodontids have been discovered in the Southern Hemisphere (Fig. 2), and the material described here establishes a new southern limit for their occurrence (indeed, for the occurrence of any Cretaceous pterosaur).

In *Pteranodon*, which attained a wing-span of about 7 m, the maximum dorso-ventral height of the scapulocoracoid is equivalent to some 5% of the total wing-span (calculated from data in ref. 2). The Toolebuc scapulocoracoid is only slightly damaged and probably had a maximum height of about 10.7 cm when complete. We estimate, by comparison with *Pteranodon*, that this scapulocoracoid was derived from a pterosaur with a wing span of about 2 m.

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Cortical binocularity in infants

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The primate visual cortex, including that of man, receives separate input from each eye and these interact in binocular cortical neurones. This organization is known to be vulnerable to disruption in early life¹. To understand the development of human visual cortex, and to detect and assess disorders of binocular function at the earliest possible age, a robust method is needed for detecting binocular interactions in the infant's visual system. We have done this by recording cortical visual evoked responses (VERs) to the onset and offset of binocular correlation in a large-screen dynamic random dot display. We report here that, in general, the human infant has a functional binocular visual cortex by 3 months of age, with some individuals showing cortical binocularity at an earlier age.

We used a stimulus display which has been found to yield prominent VERs in adult subjects with normal binocular vision^{2,3} and in an alert macaque monkey⁴, and which does not produce VERs in patients whose binocular function has been impaired by monocular retrobulbar neuritis⁵. It consists of a random dot pattern generated on red and green channels of a projection video monitor (Advent 1000 A), producing a large-screen back-projected display (157 × 130 cm), with each dot 8 × 5 mm; at the infant's viewing distance of 40 cm this gave dots 1.1 × 0.7 degrees in a display 126 × 117 degrees. The stimulus alternates between a correlated phase, in which the dot patterns are identical in the red and green channels, and an anti-correlated phase in which a dark dot in the green channel corresponds to every bright dot in the red and vice versa. Because a fresh random dot pattern is generated for each TV frame (that is, at 30 Hz), the alternation between these phases is not detectable in either channel viewed alone. When the channels are displayed to the two eyes separately, by means of red and green Wratten filters over the projection lenses and corresponding filters in lightweight goggles worn by the infant, the stimulus alternation can only be detected by binocular interaction, that is, it is 'strongly cyclopean' (ref. 6). The use of correlation/anti-correlation has the merit for infant testing that the binocular relationship is not affected by head tilt. At slow rates of alternation, the correlated phase appears to an adult as dynamic noise on a well-defined flat surface, whereas the anti-correlated phase appears rivalrous and incoherent in depth. In preliminary experiments we found that an alternation rate of 1.9 Hz was optimal for VER recording. At this rate it was difficult to differentiate the two alternating percepts, but a clear pulsation of the display at the alternation frequency was visible with binocular viewing.

Bipolar VERs were recorded from a pair of Grass gold electrodes 1 cm above theinion and at the vertex, with an indifferent electrode on the forehead. Interelectrode resistances between 5 and 20 kΩ were generally attained. Potentials were amplified with a passband of 0.25–15 Hz and cumulated in a signal averager with a 1.024 s sweep, each sweep containing two

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Table 1 Proportion of infants showing significant VERs with cyclopean stimulus, compared with control conditions

Age range*	No. of infants in group	Number of infants showing cyclopean VER
4 weeks–2 months	9	4
3–5 months	9	9
5–8 months	8	6

* There were no infants in the age range 10–13 weeks inclusive in the sample.

cycles of the stimulus alternation. The averager automatically rejected sweeps containing high-amplitude artefacts, and averaging was manually interrupted when the infant was drowsy, agitated, making gross movements, or looking away from the display. (The success of the experimenter holding the infant in minimizing these states is critical to the success of the procedure.) The cumulated evoked response was plotted after 25, 50, 75, and 100 sweeps, and the waveforms were digitized for analysis.

Subjects were healthy infants attending the paediatric clinic at Mount Sinai Hospital, and relatives of ophthalmological patients, and ages ranged between 4 and 36 weeks. They were tested when calm and alert, as far as possible, although it was more difficult to sustain this state through preparation and testing in the younger infants, especially following a long clinic attendance.

Five stimulus conditions were used for VER recording: (1) 'Contrast': when viewed without goggles, the display appears to alternate between a yellow-black (correlated) and a red-green (anti-correlated) dot pattern. This served to check that we could record VERs from a particular subject. (2) 'Red-green': viewed with goggles so that any VER recorded was due to activation of binocular cortical neurones. (3) 'Red-red' control: with goggles so that each eye saw the same channel. Any VER recorded here must be due to crosstalk between the channels, visibility of contrast changes round the edge of the goggles, or electrical artefact. (4) 'Misaligned control': correlation and anti-correlation were both destroyed by misaligning the patterns vertically by 10 degrees; again any VER is an indicator of crosstalk or artefact. (5) 'Looking away control' in which the infant was directed away from the display—this served principally as a control for the contrast condition. It was not possible to run all conditions on all infants, but in all the 27 infants whose data are reported, recordings were obtained in contrast, red-green, and one or more of the control conditions. In only five cases was it necessary to compare the red-green with the weakest, 'looking-away' control.

Figure 1 shows VERs recorded successively from a 2-month infant. In (a) a VER waveform at twice the frequency of the correlated/anti-correlated alternation clearly emerges in the course of 100 sweeps, (b) the patterns displayed to the two eyes are misaligned vertically and the VER is completely absent. In (c) the patterns are realigned and the VER reappears, (d) a single channel is displayed to both eyes and the VER is again absent. This infant therefore showed VERs which can only be a cortical response to binocular correlation.

In many infants the VER records were not so noise-free as in Fig. 1 (although amplitudes were generally greater—of the order of 10 μ V). We therefore adopted a statistical procedure for confirming the presence of a VER that cumulated over sweeps in the 'red-green' condition and was absent in controls.⁷ The amplitude of a constant VER signal increases linearly with the number of sweeps which are cumulated, whereas noise amplitude, present in both test and control runs, increases as the square root of the number of sweeps. For each run we obtained a record of the cumulated signal after 25, 50, 75 and 100 sweeps. Each of these records was digitized, and its products computed with sine and cosine functions at the stimulus alternation frequency and its second harmonic, to give the amplitude and

phase of components at these frequencies in the waveform. Regression lines were computed for the amplitude of each component as a function of number of sweeps for the records from a single run. It was taken as positive evidence for a binocular VER if the slope of the regression line for either component for a 'red-green' run was statistically significantly greater (at the $P < 0.05$ level) than the slope for a control run.

The group of infants tested were divided by age into three groups. Table 1 shows the number of infants in each group from whom positive evidence of binocular VERs was obtained. All the infants who gave negative results showed satisfactory VERs in the contrast condition.

The VERs found could be either at the first or the second harmonic of the stimulus frequency. Generally, but not invariably, the VER obtained in the 'red-green' binocular condition was the same harmonic as that obtained with the same individual in the contrast condition.

In addition to the 27 infants included in Table 1, one 17-week infant with congenital esotropic strabismus was tested both before and the day after corrective surgery, and on both occasions this child showed a VER in the contrast condition but not in 'red-green' binocular viewing. It is too soon to know whether subsequent development of binocularity will be possible in this case, or whether the lack of binocularity was congenital and permanent.

We conclude that, at least by the age of 12 weeks, the normally developing human visual cortex contains neurones which are activated by binocularly correlated stimuli. Such neurones are clearly present in some younger infants. We do not know whether the failure to elicit VERs in half the infants under two months indicates a true absence of functional binocularity, or simply the more labile attentional state of these younger infants. It is possible, but in our view unlikely, that the apparent lack of binocularity in some younger infants could have been due to errors of convergence⁸. We believe that the failure to

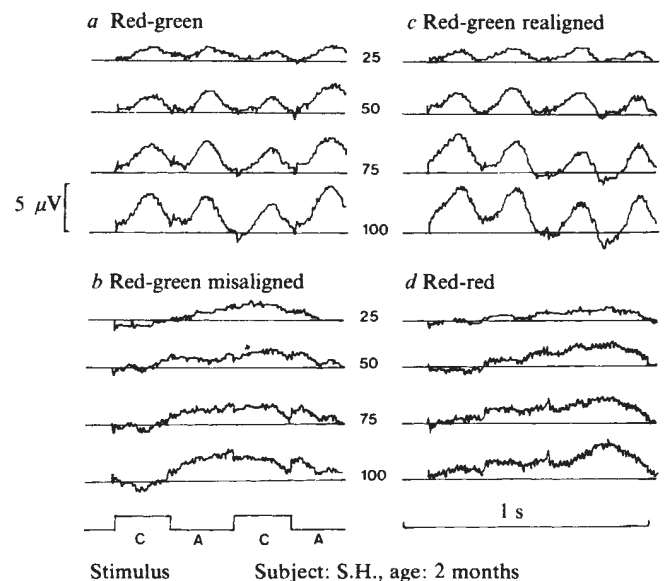


Fig. 1 Visual evoked responses (VERs) recorded from a 2-month infant using a 'cyclopean' stimulus: (a) the infant wore 'red-green' goggles so that the two eyes saw different pattern components. The stimulus trace (bottom left) shows the alternation between binocular correlation (C) and anti-correlation (A): a VER synchronized with the stimulus changes is clearly present; (b) when a vertical misalignment of the two eyes' patterns was introduced the VER disappeared; (c) the alignment of the patterns was restored and the VER reappeared; (d) when both eyes see the same pattern component (through 'red-red' goggles) no VER is present. The four traces for each condition represent the cumulated signal after 25, 50, 75 and 100 sweeps within a single run. Any true VER will appear with progressively increasing amplitude in such a series.

demonstrate binocular VERs in two infants of the oldest group can be ascribed to the very noisy signals obtained from these more active individuals.

Other behavioural investigations have tested whether infants can discriminate areas of stereoscopic disparity within a static or dynamic random dot stereogram^{9,10}. The most extensive of these⁹ found evidence for stereoscopic discriminations in 3½-month but not in younger infants. It is possible that an infant could have cortical neurones which responded to binocular correlation in our experiment, but which did not respond differentially to binocularly correlated patterns with varying disparities. Binocular cells without any fine degree of disparity tuning have been reported in the immature visual cortex of the cat¹¹. Further behavioural and evoked potential studies, which compare the random dot correlograms used here with random dot stereograms, are required to test whether the binocularity found in at least some infants under 3 months is of this nature. In

adults, both dynamic random dot correlograms (the stimulus used in this study) and stereograms yield large VERs, but of different shapes².

Animal^{1,12} and human¹³ studies have shown that binocular organization is vulnerable to environmental modification, implying that early correction of binocular disorders such as strabismus may be critical. Our demonstration of cortical binocularity in normal 3-month-old infants opens the possibility that this robust VER method could be used to diagnose and assess early abnormalities of binocular development and effects of treatment.

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Bloom-forming cyanobacterium *Microcystis aeruginosa* overwinters on sediment surface

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Cyanobacteria (blue-green algae) commonly occur in the phytoplankton of lakes and reservoirs and sometimes develop as blooms which can cause deoxygenation, toxin production and nuisance odours¹. The factors which govern the occurrence and seasonal development of such blooms in surface waters are imprecisely understood and little is known about the origins of the bloom-forming populations within particular water bodies (see, however, refs 2, 3). Using ¹⁵N as tracer, we show here that the appearance of *Microcystis* in the phytoplankton of an experimental enclosure in the summer of 1977 correlated directly with the presence of particulate ¹⁵N which could only have been sediment-derived and which originated mainly from *Microcystis* cells deposited on the sediment surface the previous year. The most plausible explanation is that *Microcystis* overwinters on the sediment surface and by so doing provides an inoculum of colonies from which the epilimnetic population develops the following summer^{4–8}. Possible mechanisms which allow the mass recruitment of *Microcystis* to the plankton are considered elsewhere^{2,3}.

On 19 August 1976, 11 kg NaNO₃ (enriched with 9.63 atom% excess ¹⁵N) and 789 g KH₂PO₄ (N:P atomic ratio, 10:1) were dissolved and sprayed onto the surface waters of an 18,500 m³ experimental enclosure (45.7 m diameter, 12 m deep) in Blelham Tarn in the English Lake District when the waters were thermally stratified¹⁰.

When the fertilizer was added, algal biomass in the water column was low (<5 µg chlorophyll *a* per l) but within 14 days an extensive bloom dominated by the cyanobacterium *Microcystis aeruginosa* developed in the epilimnion. In October,

stratification broke down and the particulate ¹⁵N in the water column (7.2 kg N enriched with 1.31 atom% excess ¹⁵N) sedimented out (Fig. 1). Light microscopy showed the sedimenting material to be almost entirely (>90% by volume) *Microcystis* colonies. This was deposited on the sediment surface as a layer approximately 1.0 cm thick and clearly visible in sediment core samples. On 19 February 1977 the layer of *Microcystis* still dominated the sediment surface and the cells then had a ¹⁵N enrichment of 1.33 atom% excess ¹⁵N; only 7.5% (9 g ¹⁵N) of

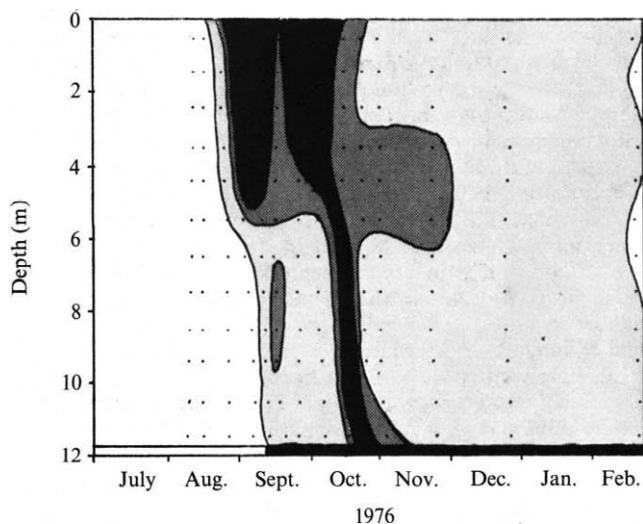


Fig. 1 The distribution of particulate ¹⁵N (total particulate N × atom% excess ¹⁵N enrichment) in the water column and sediment of a LUND enclosure (enclosure A) in Blelham Tarn, English Lake District from July 1976 to February 1977. ■, >8; ▒, 5–8; □, 1–5; □, 0–1 (all µg per l). Water samples were collected using a 5 l Friedinger bottle; sediment core samples were obtained using a Jenkins surface sediment corer¹², particulate material was separated by filtration onto precombusted Whatman GF-C disks; organic C and total N were measured using a Hewlett Packard 185B elemental analyser or by Kjeldahl digestion. Isotope analyses were carried out on Kjeldahl distillates after conversion of the NH₄⁺ to N₂ using LiOBr (ref. 13). Isotope ratios were determined using a VG Micromass MM601 mass spectrometer.

the total isotope present in the sediment had been mineralized. The experiment therefore provided a labelled sediment population whose subsequent fate could be followed.

The walls of the enclosure were lowered on 20 February 1977 to equilibrate the isothermal waters with those of the Tarn and thus remove ^{15}N from the water column. After 30 days the walls were raised and the ^{15}N content of the particulate and soluble material in the water column was subsequently monitored.

Figure 2a presents data on the particulate ^{15}N found in the water column from the time of isolation of the enclosure (22 March 1977) until late September 1977. It is seen that very little particulate ^{15}N was present in the water column immediately after isolation (10–100-fold lower than that present before opening the enclosure; see Figs 1 and 2a), indicative of the success of the flushing procedure. Particulate ^{15}N concentrations in the surface waters then increased to a maximum in May. As soluble ^{15}N was undetectable in the water column until August when $^{15}\text{NH}_4^+$ was first released from the sediment into the anoxic hypolimnion, the particulate ^{15}N increase must have been sediment-derived. This particulate ^{15}N increase in May corresponded with the first appearance of *M. aeruginosa* in the water column (see Fig. 2c). An epilimnetic decline in particulate ^{15}N then occurred with the onset of stratification in June.

In mid-July a very pronounced input of ^{15}N was noted below 7.5 m. Over the period 21 June–12 July this input was 230 mg ^{15}N and the net increase in the whole enclosure was 84 mg ^{15}N . That is, there was significant movement of sediment-derived ^{15}N particulate material into the hypolimnion. The integrated water column ^{15}N concentration in mid-July was 940 mg ^{15}N (1% of that present on the sediment surface). On analysing the subsequent changes in particulate ^{15}N over the period 12 July–11 August, a net decrease in particulate ^{15}N occurred, due presumably to some material sedimenting. However, of more importance, there was an input of 122 mg ^{15}N to the epilimnion. That is, the particulate ^{15}N which originated in quantity from the sediment in mid-July had reached the surface waters. In late September the particulate ^{15}N sedimented.

Figure 2b, c provides information on the nature of the particulate material in the water column. The carbon-to-nitrogen (C:N) ratio data (Fig. 2b) show that immediately after isolating the enclosure in March the particulate material had a C:N ratio of 10.4:1. This ratio then increased to a maximum of 12.2:1 in early May indicating that the particulate material had a large detrital component. A particulate ^{15}N maximum also occurred then (see Fig. 2a). In mid-July the C:N ratio changed sharply at depth to <9 indicative of the presence of phytoplankton (the C:N ratios of healthy *Microcystis* the previous year were 7–8:1) with minimum C:N values (8:1) being found where the particulate ^{15}N concentration was also highest (8.5 m; see Fig. 2a). In August the C:N ratios in the surface waters decreased to a minimum.

The abundance of *Microcystis* (Fig. 2c) was then examined and correlated with changes in particulate ^{15}N (Fig. 2a) and in C:N ratios (Fig. 2b). *Microcystis* was first detected in very low numbers in the surface waters during May 1977. These colonies failed to develop and *Microcystis* colonies present in May could not have been solely responsible for the total particulate ^{15}N noted. This May 1977 *Microcystis* (based on cell volume estimates and a ^{15}N enrichment of 1.33 atom % excess ^{15}N) accounted for <25% of the observed water column ^{15}N concentration. Together with the high C:N ratio this suggested that the labelled material was largely cell debris derived from the sediment surface. Also, at that time the colonial chrysophyte *Uroglenopsis americana*¹¹ developed. This alga, which was absent from the phytoplankton the previous year, may exist, like other chrysophytes, as cysts on the sediment surface until germination, possibly obtaining some ^{15}N from sediment pore water and then moving into the water column. In late July *Microcystis* became clearly detectable in the water column and was concentrated at 8.5 m, the site of highest ^{15}N concentration. In August, *Microcystis* developed in the surface waters and its presence there correlated with the presence of particulate ^{15}N

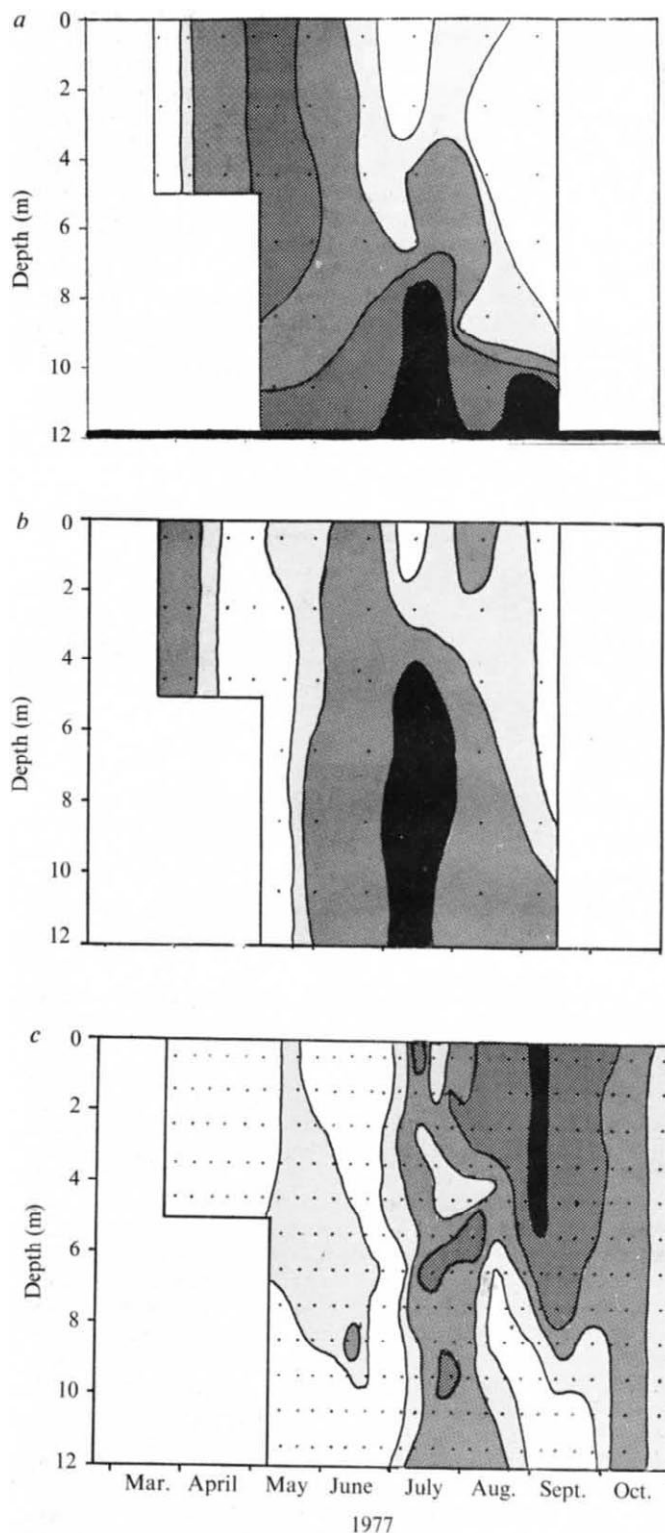


Fig. 2 a, The distribution of particulate ^{15}N in the water column and sediment of a Lund enclosure in Blelham Tarn from March 1977 to September 1977. ■, >80; ■, 50–80; ■, 35–50; □, 25–35; □, 0–25 (all ng per l). b, The distribution of particulate organic carbon-to-nitrogen ratio of water column material of a Lund enclosure in Blelham Tarn from March 1977 to September 1977. □, >12:1; □, 12:1–11:1; ■, 11:1–9:1; ■, <9:1. c, The distribution of *M. aeruginosa* colonies in the water column of a Blelham Tarn enclosure from March 1977 to October 1977. ■, >7; ■, >3; ■, 1–3; □, 0.1–1; □, <0.1 (per ml). The colonies were counted by sedimentation and microscopy¹⁴.

(the *Microcystis* biomass then could easily account for the total particulate ^{15}N in the epilimnion) although, because of its undoubted assimilation of unlabelled nitrogen during growth, the ^{15}N enrichment of *Microcystis* decreased. In late September *Microcystis*, like the particulate ^{15}N , sedimented.

The above data collectively provide evidence that *Microcystis* overwinters on the sediment surface and serves as an inoculum for the following year's epilimnetic growth.

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Explanation of sterility in $t^x t^y$ male mice

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The lethal ' t alleles' of the T complex on mouse chromosome 17 (refs 1, 2) have been assigned to six complementation groups (the definitive classes being t^0 , t^9 , t^{12} , t^{w1} , t^{w5} and t^{w32}), each causing embryonic death at a particular stage. Intercomplementation heterozygotes ($t^x t^y$) show 'partial' complementation, so that only some $t^x t^y$ offspring survive^{3,4}. These survivors appear morphologically normal; indeed the females, being fertile, seem entirely normal. The $t^x t^y$ males, however, are sterile. Among the reasons given for this sterility in some $t^x t^y$ males are low motility of the $t^x t^y$ spermatozoa⁵, and an inability to effect fertilization either *in vitro* or *in vivo* even when already present at the site of fertilization^{6,7}. Spermatozoa from sterile $t^x t^y$ males were reported to be apparently present in 'normal' numbers in the uterus, although actual ejaculate numbers have not been quoted for these, or indeed for normal fertile males^{5–8}. In the human, typical ejaculate counts of less than 20×10^6 spermatozoa, where the normal ejaculate contains $100\text{--}250 \times 10^6$, are judged to indicate clinical sterility⁹. The comparison of numbers of spermatozoa¹⁰ ejaculated into the female tract by sterile $t^6 t^{w5}$, $t^6 t^{w32}$ and $t^{w5} t^{w32}$ (all 'strong' mutations²) and control mice clearly shows an even greater oligozoospermia (about 100 times less than controls) occurring in the $t^x t^y$ males.

Balanced lethal stocks of Tt^6 , Tt^{w5} and Tt^{w32} mice are maintained in the Department of Zoology at the University of Birmingham; $t^x t^y$ tailed males were produced from crosses between these tail-less stocks ($Tt^x \times Tt^y$). I investigated the numbers of spermatozoa ejaculated by sterile $t^x t^y$ and control males by mating them with females at times ranging from 2 h before until 4 h after ovulation. Ovulating ++ (C3H), T+, +t and Tt females aged 2–10 months were used. (No differences were observed between results from induced or naturally ovulating females of differing ages or genotypes.) Females were killed as soon as a vaginal plug was seen (30 min observations). Control females were artificially inseminated with known

numbers of spermatozoa. The uterus and 'plugged' vagina were removed complete, after the ovarian end of the uterine horns had been ligatured, into a small (50 mm) Petri dish with as little blood as possible, and flushed with phosphate-buffered saline (PBS, Oxoid Dulbecco A) from a syringe and 21-gauge needle. The uterus was cut open, washed and scraped gently to transfer all spermatozoa to the wash-medium. All medium was taken up into 10 ml PBS and this was centrifuged at 250g for 15 min. The pellet was resuspended by gentle agitation into a further 10 ml PBS; 0.02-ml drops of this suspension were spotted out and air-dried on clean, marked slides. The dried spots were stained for 1 min in Wells and Awa stain¹¹, air-dried and mounted in DPX (Hopkins and Williams Chemicals). All spermatozoa in each spot were counted in order to calculate the number in the 10 ml PBS, which was assumed to be the number ejaculated. When a visual estimate suggested a count greater than 1,000 per drop, the spermatozoa in 10 random $\times 40$ objective fields (area 0.16 mm^2) were counted and used to calculate total numbers in the dried drop of known size. The area of the spot was measured either by taking two diameter measurements at 90° where the spot was circular, or by making a trace on fine graph paper to calculate the area where the spot was misshapen. This was done to ensure standard treatment of all samples. I checked the multi-field count method by further diluting sperm suspensions and counting every sperm in each spot (diluted to contain 100–1,000 spermatozoa per drop). That gave numbers in agreement with the counts from the spots of lesser dilution (<10% error). Table 1 shows the recovery of artificially inseminated spermatozoa, and makes possible estimation of the efficacy of the flushing technique; Table 2 shows the recovery of spermatozoa from females mated to control and $t^x t^y$ males.

Table 1 Assessment of efficacy of the flushing technique

	Spermatozoa artificially inseminated log $\pm$ s.d. of counts (linear value $\times 10^6$)		Spermatozoa recovered log $\pm$ s.d. of counts (linear value $\times 10^6$)
Male			
$+t^6$	6.78 ± 0.12 (6.28 ± 2.0)	(1)	6.50 ± 0.11 (3.23 ± 0.8)
		(2)	6.80 ± 0.03 (6.30 ± 0.4)
		(3)	6.66 ± 0.18 (4.83 ± 2.0)
Tt^{w5}	6.36 ± 0.10 (2.34 ± 0.6)	(1)	6.33 ± 0.14 (2.21 ± 0.7)
		(2)	6.38 ± 0.12 (2.50 ± 0.7)
$+t^{w32}$	6.34 ± 0.09 (2.23 ± 0.5)	(1)	6.09 ± 0.25 (1.39 ± 0.9)
		(2)	6.49 ± 0.08 (3.09 ± 0.6)
		(3)	5.98 ± 0.16 (1.00 ± 0.3)

Spermatozoa from these control males were stripped from vasa deferentia and epididymides into warm (37°C) PBS. 0.05–0.10-ml sperm suspension was carefully injected per cervix as soon as possible (5–8 min) into etherized, oestrous females, and samples were diluted for counting to estimate the number of spermatozoa injected per female. Females were killed within 10 min of insemination, by cervical dislocation, and the uteri were exposed. Cervices were clamped and the uteri flushed as described in the text. Sperm numbers are given as log means $\pm$ s.d. as recommended by Cohen^{10,12}, and the linear values are also presented.

Recovery of spermatozoa from artificially inseminated females was relatively successful considering the difficulty of attempting to deposit known numbers of spermatozoa into the uterus. On the worst interpretation of the figures, recovery was usually 85% (the very worst was 43%). So the estimates of numbers of ejaculates from control and $t^x t^y$ mice can be assumed to be at least as representative. The numbers assessed for C57BL males are close to previous estimations¹², but are higher than those obtained by electroejaculation¹⁴. The figures for the $t^x t^y$ mice of all three types are very variable between males, and even, to some extent, between copulations by one

Table 2 Recovery of spermatozoa from females mated with $t^x t^y$ and control males

$t^x t^y$ sterile males			Control males		
Male	No. of matings	Sperm count log mean $\pm$ s.d.* (linear value)	Male	No. of matings	Sperm count log mean $\pm$ s.d.* (linear value $\times 10^6$)
$t^6 t^{w5}$	4	4.30 $\pm$ 0.25 (22,500 $\pm$ 12,000)	$Tt^{w32\pm}$	1	6.96 $\pm$ 0.04 (9.13 $\pm$ 0.9)
$t^6 t^{w5}$	4	5.10 $\pm$ 0.53 (209,000 $\pm$ 244,000)	$Tt^{w32\pm}$	1	6.77 $\pm$ 0.09 (5.95 $\pm$ 1.1)
$t^6 t^{w5}$	3	<3.00 (<1,000)†	$Tt^{w5\pm}$	2	6.85 $\pm$ 0.35 (8.35 $\pm$ 6.0)
$t^6 t^{w5}$	4	<3.00 (<1,000)†	$Tt^{w5\pm}$	2	7.11 $\pm$ 0.08 (13.00 $\pm$ 2.5)
$t^6 t^{w5}$	2	<3.00 (<1,000)†	$T+$	1	7.30 $\pm$ 0.05 (19.82 $\pm$ 1.9)
$t^6 t^{w32}$	5	4.99 $\pm$ 0.30 (113,000 $\pm$ 54,000)	$T+$	1	6.44 $\pm$ 0.13 (2.82 $\pm$ 0.8)
$t^6 t^{w32}$	1	3.79 (7,000)†	+ + C57BL	1	7.14 $\pm$ 0.06 (13.87 $\pm$ 1.8)
$t^{w5} t^{w32}$	2	4.88 $\pm$ 0.51 (100,000 $\pm$ 98,000)	+ + C57BL	2	6.86 $\pm$ 0.04 (7.17 $\pm$ 0.6)
			+ t^{w5}	1	7.23 $\pm$ 0.06 (16.77 $\pm$ 2.5)

Female mice were either induced to ovulate (5 IU follicle stimulating hormone followed 48 h later by 5IU human chorionic gonadotropin; ovulation assumed to occur 13 h later¹³), or used when naturally ovulating as assessed by vaginal smear test. $T+$ and $+t$ mice were obtained as F_1 from C57BL $\times$ Tt crosses. The $t^x t^y$ males were considered sterile if they were run with five known-fertile females for 4 weeks or more without pregnancies (five 'mating units').

* Standard deviations of pooled counts per mating where the s.d. of each count <20% of mean; except where only one count is given, then the s.d. of that count is shown.

† These counts were based on very few (<20) spermatozoa per spot.

‡ Known fertile control males.

male. However, they are at least two orders of magnitude lower than the figures from the control males. In the human just one order of magnitude difference seems to confer a state of clinical sterility, so this hundredfold difference would adequately explain the sterility of these $t^x t^y$ males.

In addition to the other suggested causes of the sterility⁵⁻⁷, the extreme $t^x t^y$ oligozoospermia reported here presents a convincing explanation; it is tempting to consider this as the chief cause, with reduced motility and lack of fertilizability being secondary phenotypic expressions arising from the infertile state¹⁵. In this simple solution to the $t^x t^y$ sterility it is not necessary to invoke altered sperm surface antigens interacting within the female¹⁶; the answers appear to lie in the course of spermatogenesis, which is in accordance with and extends the suggestion¹⁷ that events before mating affect $t^x t^y$ fertility. Preliminary studies so far indicate fewer spermatozoa present in the epididymides and vasa deferentia of $t^x t^y$ males compared with controls¹⁸. It is to be hoped that further work will elucidate the actual processes resulting in this severe oligozoospermia found in these heterozygous $t^x t^y$ male mice.

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Molecular analysis of the genetic relationship of *trans* interacting factors at the T/t complex

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The T/t complex is an extensive genetic region proximal to the H-2 complex on mouse chromosome 17, with multiple effects on embryonic development, spermatogenesis and recombination¹⁻³. Recently, two-dimensional gel analysis of testicular cell proteins identified a gene within the T/t complex that codes for a major cell surface-associated protein, p63/6.9 (ref. 4). The wild-type gene, *Tcp-1*⁺, codes for a 63,000-molecular weight protein (p63/6.9b), whereas a mutant allele, *Tcp-1*⁻, which occurs in all intact *t* haplotypes, codes for a more acidic form of the protein (p63/6.9a). Analysis of partial *t* haplotypes obtained from rare recombination events showed that *Tcp-1*⁻ correlated completely with the tail interaction factor *t*^T, which is thought to be a genetic allele of *T*, thus raising the possibility that the locus of *T* codes for the p63/6.9 protein. We report here that the p63/6.9 proteins produced by seven chromosomes carrying independently derived dominant mutations at the locus of *T* are all indistinguishable from the wild-type form; thus, the cumulative data indicate that the *Tcp-1* gene is most probably not at the locus of *T*.

The T/t complex was originally defined by the dominant mutation *Brachyury* (*T*), which shortens the tails of heterozygotes and is lethal at 10.5 days of gestation when homozygous. Recessive lethal *t* haplotypes are found at high frequency in wild mice and are identified by their interaction with *T* to produce a tailless phenotype. Recombination data demonstrate that naturally occurring *t* haplotypes are separable into at least two well defined genetic factors (see Fig. 1): a proximal factor, *t*^T, is allelic to and interacts in *trans* with *T* to produce taillessness; a distal factor, *t*^{lethal}, results in embryonic death when it is homozygous. Other data indicate that the *t* haplotype may be

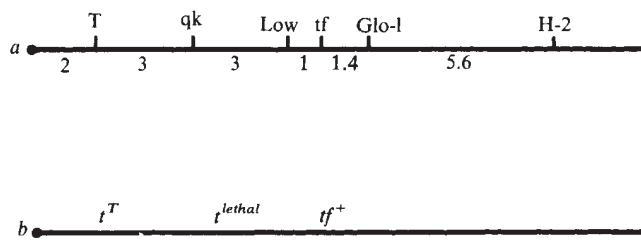


Fig. 1 a, Mouse chromosome 17 and relevant markers. Genetic distance between markers is given in centimorgans. b, t Haplotype showing the separation of t factors.

separated into several other factors including those factors responsible for male sterility and distorted segregation ratios⁵.

Partial t haplotypes, recovered from recombination events, were used to map the *Tcp-1* gene: *Tcp-1*^a was not associated with the one available partial haplotype (t^{h17}) carrying only the t^{lethal} factor, but correlated completely with the presence of the tail interaction factor, t^T , in a variety of partial haplotypes examined. As t^T and T behave as genetic alleles, the simplest explanation for the data was that the p63/6.9 protein was the direct gene product of the locus of T . However, the form of p63/6.9 produced by the one T mutation examined (*Brachyury*) was indistinguishable by two-dimensional gel analysis from wild type.⁴ Nevertheless, as two-dimensional gel analysis is capable of defining only about one-third of all point mutations, the possibility remained that the locus of T codes for the p63/6.9 protein.

Seven independently derived dominant tail mutations at the locus of T have been analysed here (Table 1). These include two radiation-induced mutations and five spontaneous mutations which occurred in laboratory stocks. Of the five spontaneous mutations at T , two seem to be deletions of a portion of chromosome 17 including the T locus and another locus, *quaking* (*qk*). All T -locus mutations meet the following criteria: (1)

they are dominant mutations mapped to the T/t -complex region of chromosome 17, and are lethal when homozygous; (2) they interact with t to produce a tailless phenotype in T/t individuals; (3) they fail to complement one another and hence T^x/T^y individuals die during embryonic development.

The expression of the p63/6.9 protein in heterozygotes for seven independent T -locus mutations was analysed by two-dimensional gel electrophoresis of radio-labelled testicular cell proteins (Fig. 2, Table 2). Animals which carried T , T^{hp} , T^{wis} , T^J , T^c and T^{OR4} opposite a chromosome with a wild-type *Tcp-1*^b allele expressed a distinct p63/6.9b protein spot, but showed no additional spots (relative to known *Tcp-1*^b homozygotes) in the p63/6.9 region of two-dimensional gels. In each case, this result indicates that the chromosome opposite *Tcp-1*^b either expresses p63/6.9b or does not express any form of the p63/6.9 protein.

To distinguish between these two possibilities, we analysed the protein patterns of mice carrying each of these mutations opposite a chromosome carrying a *Tcp-1*^a allele. Animals which carried T , T^{wis} , T^J , T^c and T^{OR4} and a *Tcp-1*^a allele all expressed both p63/6.9b and p63/6.9a. Therefore, each of these T -locus mutations expresses the wild-type form of the p63/6.9 protein. As described previously, mice with a $T^{hp}/Tcp-1$ ^a genotype express only p63/6.9a (ref. 4). Therefore, chromosomes with the T^{hp} deletion do not have a functional *Tcp-1*^a gene.

The apparent deletion T^{Or1} was analysed for *Tcp-1* gene expression. $T^{Or1}/Tcp-1$ ^a mice express both p63/6.9b and p63/6.9a. This indicates that the T^{Or1} deletion does not cover the *Tcp-1* gene. However, $T^{Or1}/Tcp-1$ ^b mice also express both p63/6.9b and p63/6.9a. These data imply that the T^{Or1} chromosome, far from containing a deletion of *Tcp-1*, actually possesses both the *Tcp-1*^b and *Tcp-1*^a alleles. Further analysis of this chromosome is in progress.

The *Tcp-1* gene codes for a major cell surface-associated protein, p63/6.9. Two structural alleles of this gene have been

Fig. 2 A, Complete two-dimensional fluorograph of Nonidet P40-soluble proteins from T^c/t^{w73} testicular cells. b, p63/6.9b; a, p63/6.9a. The basic side of the gel is on the left. B-E, The p63/6.9 regions of fluorographs are shown for the following genotypes: B, $T^{wis}/+$; C, T^{wis}/t^{w73} ; D, $T^{OR4}/+$; E, T^{OR4}/t^{w18} . Testicular cells were isolated essentially as described by Romrell *et al.*¹² as modified by Silver *et al.*⁴. Cells were labelled in medium containing 200–250 $\mu\text{Ci ml}^{-1}$ ³⁵S-methionine, for 4–5 h at 34 °C. Cells were washed and lysed at a concentration of 10^7 cells ml^{-1} with phosphate-buffered saline containing 1% Nonidet P40, 2 mM methionine and 1 mM phenylmethylsulphonyl-fluoride. Nonidet P40-soluble proteins were obtained after centrifugation at 15,000g at 4 °C for 60 min and were stored at –80 °C. Proteins were separated according to the procedure described by O'Farrell and O'Farrell¹³ as modified by Silver *et al.*⁴.

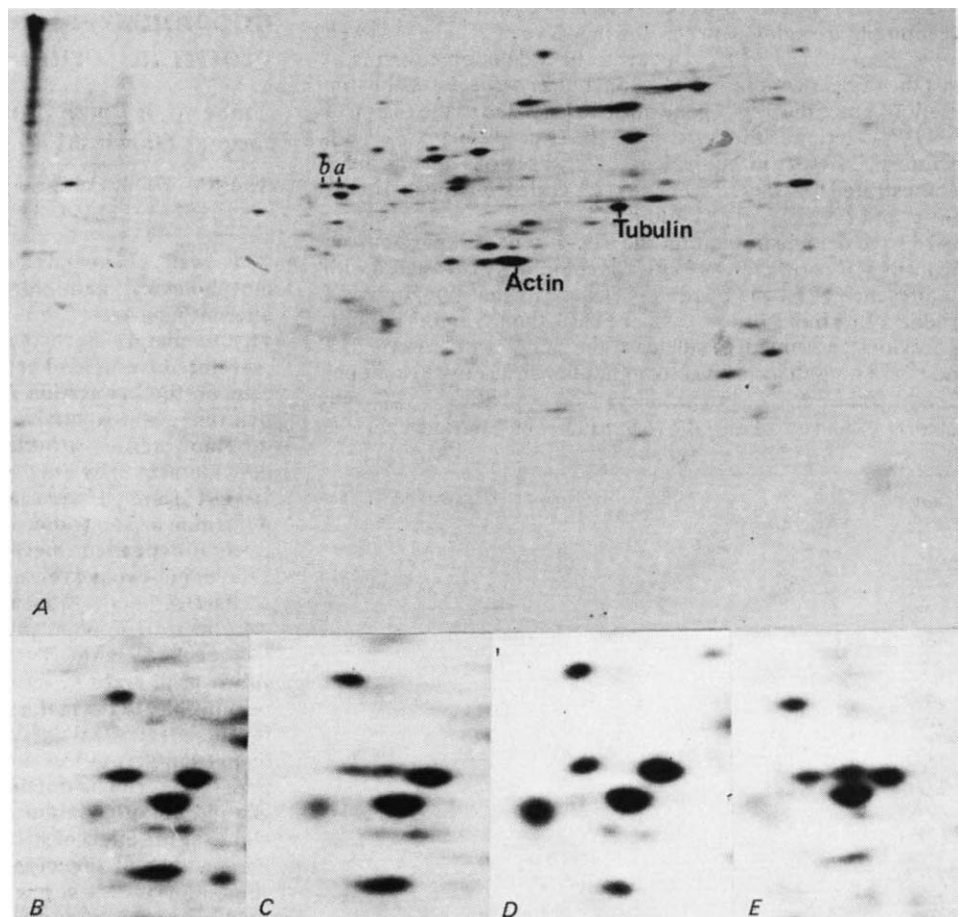


Table 1 Origin of the various *T* mutations

<i>T</i> Mutation	Origin	Ref.
<i>T</i> <i>Brachyury</i>	Spontaneous in laboratory stock	7
<i>T^{hp}</i> <i>T^{hairpin}</i>	Spontaneous in an AKR mouse	8
<i>T^{Orl}</i> <i>T^{Orleans}</i>	Spontaneous in a Swiss/Orleans mouse	9
<i>T^{Wis}</i> <i>T^{Wisconsin}</i>	Spontaneous in laboratory stock	Moutier (personal communication) D.B.
<i>T^J</i> <i>T^{Jackson}</i>	Spontaneous in a BALB/CHu mouse	(unpublished data) Hummel (personal communication)
<i>T^c</i> <i>T^{curtailed}</i>	Radiation induced	10
<i>T^{OR4}</i> <i>T^{Oak Ridge4}</i>	Radiation induced	11

identified. All wild-type chromosomes carry *Tcp-1^b* whereas all naturally occurring *t* haplotypes carry *Tcp-1^a*. As *Tcp-1^a* and the *t^T* allele of the *T* locus seemed to be completely correlated⁴, we sought to determine whether the *Tcp-1* gene and the locus of *T* were identical. Of seven independently derived *T*-locus mutations examined, six allowed expression of a form of p63/6.9 which seems to be identical to the wild-type p63/6.9. The seventh, *T^{hp}*, acted as a null allele of *Tcp-1* (*Tcp-1^a*).

At least 33% of all single base pair substitutions will result in an alteration in protein charge that can be readily detected by standard isoelectric focusing techniques⁶. If we take a conservative estimate, 66% of all single base pair substitutions go undetected (that is, those resulting in a neutral-to-neutral amino acid change). If we assume that the *Tcp-1* gene is at the locus of *T*, the probability that in six independent mutational events at *T* such a shift has occurred and that it remains undetected is (0.66)⁶. The probability of detecting at least one such shift is 1 - (0.66)⁶ or 92%. Because, in fact, no shifts were detected, we can say with 92% probability that the *Tcp-1* gene is separate from the locus of *T*. This view is compatible with other genetic evidence. *T^{hp}* is known to be a deletion covering at least the 3-centimorgan region between and inclusive of *T* and *qk* as well as the locus of *Tcp-1*. *T^{Orl}* seems to be a deletion covering at least the same 3-centimorgan region, but *Tcp-1* is not within the deletion. Thus, the *Tcp-1* gene must be localized to the region of the *T^{hp}* deletion either proximal to *T* or distal to *qk* and, therefore, cannot be equivalent to *T*. Previous results have demonstrated that the *Tcp-1* gene is separate from the loci of *qk*, *low*, *tf* and *t^{lethal}* (see Fig. 1 and ref. 4).

We have demonstrated that the *Tcp-1* gene is separate from the locus of *T*; however, as *Tcp-1^a* is completely correlated with the presence of *t^T*, the possibility still exists that p63/6.9a is a product of *t^T*. If this proves to be the case, then *T* and *t^T* cannot, as previously assumed, be alleles at the same locus. Although *T* and *t^T* are not separable by recombination, one must remember

that one of the effects of *t* haplotypes is the suppression of recombination along chromosome 17. *T* and *t^T* interact in *trans* to produce taillessness, but it is possible that *trans* interactions can occur even between two interacting factors at separate loci. Until the precise relationship between *t^T* and *Tcp-1* can be determined, one cannot comment on the validity of the assumption that *T* and *t^T* are alleles at the same locus.

Although it is possible that the p63/6.9 protein may be associated with some other property of the *T/t* complex, such as segregation distortion or sterility, for which others had suggested factors existed⁵, our data do not fit the distribution of any known *T/t*-complex factor other than *t^T*.

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Effect of striatal cells on *in vitro* maturation of mesencephalic dopaminergic neurones grown in serum-free conditions

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It is well documented that target cells can regulate the morphological and biochemical development of peripheral afferent neurones^{1,2}, but little is known about the existence of such regulatory mechanisms in the central nervous system. We therefore investigated previously the influence of striatal target cells on the maturation *in vitro* of nigrostriatal dopaminergic neurones, which survive in culture for more than 5 weeks, develop dense arborizations and both take up ³H-dopamine (DA) by a high-affinity specific process and synthesize ³H-DA from ³H-tyrosine³. Furthermore, depolarization by potassium or veratridine stimulates the release of DA through a calcium-dependent mechanism and tetrodotoxin prevents the veratridine-evoked release of the transmitter⁴. Both the number of ³H-DA uptake sites and the capacity for ³H-DA synthesis were at least doubled when the neurones were cultured with target cells from the striatum³. To determine whether glial cells which proliferate in serum-complemented medium are partly responsible for the maturation of dopaminergic neurones and/or for the effect of striatal cells, we have now repeated the experiment using serum-free medium in which virtually pure neuronal populations can be obtained⁵⁻⁷. The reduction in the number of glia did not affect either the maturation of dopaminergic cells alone, or the effect of striatal cells. Autoradiographic analysis of the number of dopaminergic cells strongly suggests that the stimulatory effect is related to increased capacities of ³H-DA uptake and synthesis per dopaminergic neurone.

Table 2 Summary of the p63/6.9 pattern of expression for the various *T^x*

Genotype	p63/6.9b	p63/6.9a
<i>T</i> /+	+	-
<i>T</i> / <i>t</i>	+	+
<i>T^{hp}</i> /+	+	-
<i>T^{hp}</i> / <i>t</i>	-	+
<i>T^{Wis}</i> /+	+	-
<i>T^{Wis}</i> / <i>t</i>	+	+
<i>T^J</i> /+	+	-
<i>T^J</i> / <i>t</i>	+	+
<i>T^c</i> /+	+	-
<i>T^c</i> / <i>t</i>	+	+
<i>T^{OR4}</i> /+	+	-
<i>T^{OR4}</i> / <i>t</i>	+	+
<i>T^{Orl}</i> /+	+	+
<i>T^{Orl}</i> / <i>t</i>	+	+

Table 1 Composition of various serum-free media and their effects on dopaminergic cell survival

	Medium 1	Medium 2	Medium 3
Basal medium	—	+	—
Proteins, hormones, salt	+	+	+
Conditioned medium	+	—	+
Survival time	≥14 days	≤10 days	≥14 days

In all cases, cells were grown on non-irradiated collagen plus polyornithine as substrate. Basal medium was as described in Fig. 1a legend; proteins, hormones, salt refer to transferrin, insulin, progesterone, putrescine, selenium salt (see text for concentrations); conditioned medium 1 was basal medium incubated for 48 h with cells (fibroblasts or total brain cells) previously grown with a serum-complemented medium for 1–4 weeks; conditioned medium 3 was medium obtained after 8 days from total brain cells directly plated and grown with medium 2.

Preliminary experiments indicated that the use of non-irradiated collagen markedly reduced the overall population of glial cells when mesencephalic cells of 13-day-old mouse embryos were cultured with serum. Furthermore, autoradiography of the cultures (Fig. 1c) after a 48-h pulse of ^3H -thymidine (during days 3–5) revealed that the number of labelled cells with large nuclei (presumptive astrocytes⁸) was only 2.3% (742 ± 109 , $n = 3$) of that found in cultures grown on UV-irradiated collagen plus polyornithine ($31,600 \pm 1,500$, $n = 3$). To eliminate glial elements further, mesencephalic cells were dissociated in a serum-free medium and then plated on non-irradiated collagen plus polyornithine in a conditioned medium obtained by 48-h incubation of the serum-free medium with monolayers of primary cultures of embryonic cells from whole mouse brain or of fibroblasts previously grown with serum. Thereafter, this conditioned medium was supplemented with $100 \mu\text{g ml}^{-1}$ transferrin (Sigma), $25 \mu\text{g ml}^{-1}$ insulin (Sigma), 2×10^{-8} M progesterone (Sigma), 6×10^{-5} M putrescine (Sigma) and 3×10^{-8} M selenium salt (Merck), and filtered (Millex; Millipore). In this medium (medium 1), the number of labelled glial cells (estimated as above) was only 0.06% (20 ± 14 , $n = 3$) of that found with serum using UV-irradiated collagen plus polyornithine (Fig. 1a, b). In medium 1, despite the virtual absence of glial cells, the overall neuronal population survived for 2–4 weeks according to the experiments (Table 1). The incubation of mesencephalic cells with exogenous ^3H -DA allowed the autoradiographic visualization of dopaminergic neurones, which exhibit numerous varicose fibres (Fig. 1d). As illustrated by Table 2, the capacities of ^3H -DA uptake and of ^3H -DA synthesis from ^3H -tyrosine increased with time in culture (up to 15 days at least). Medium 1 is thus preferable to the serum-complemented medium used in our previous study because in the latter case ^3H -DA uptake and synthesis did not increase between days 8 and 15 in mesencephalic cultures. However, as in the presence of serum³, the extent of ^3H -DA uptake or synthesis in mesencephalic cultures grown with medium 1 varied between experiments; this did not seem to be related to the cell type used to condition the medium.

When mesencephalic cells were cultured in medium 1 in the presence of striatal target cells from 15-day-old embryos (co-cultures), the glial cell population was still negligible (26.5 ± 2 , $n = 3$). Nevertheless, as revealed by the enhanced capacities of ^3H -DA uptake and synthesis in co-cultures, the striatal cells stimulated the maturation of the mesencephalic dopaminergic neurones (Table 2). These stimulatory effects varied (1.5–3 times) between experiments but were always significant, at both 8 and 15 days. These effects cannot be attributed to the higher cell concentration used in co-cultures (2.5×10^6 cells per dish) because ^3H -DA uptake in mesencephalic cultures alone is directly proportional to the number of cells plated (between 1×10^6 and 3.5×10^6 cells, results not shown).

Complementary experiments were carried out to determine whether the development of the dopaminergic neurones and their enhanced maturation in the presence of striatal cells were dependent on the occurrence in medium 1 (Table 1) of

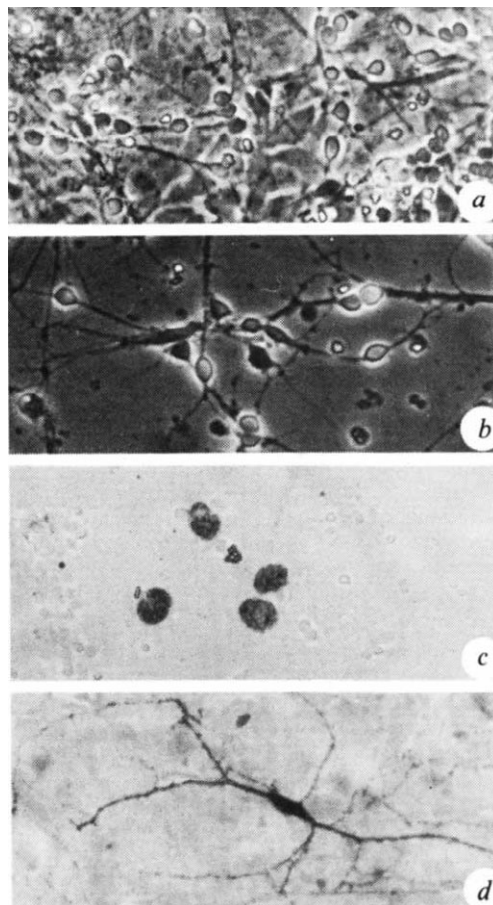


Fig. 1 a, Thirteen-day-old mouse embryonic mesencephalic cells were dissociated in a mixture of minimum essential medium and nutrient mixture F-L2 (both Gibco, 1:1) complemented with 3.3×10^{-2} M glucose, 2×10^{-3} M glutamine and 0.3×10^{-2} M sodium bicarbonate without serum (basal medium). Cells (1.5×10^6 per dish) were then plated on UV-irradiated collagen plus polyornithine and grown in basal medium plus 7.5% fetal calf serum (Irvine Scientific) as previously described¹. Arabinosylcytosine (10^{-5} M, Sigma) was added at day 5, the medium (2 ml) was replaced at day 7 and thereafter weekly. The extensive development of both neurones and glial cells at day 5 can be seen. $\times 187$. b, Mesencephalic cells were dissociated as above, plated on non-irradiated collagen plus polyornithine and grown in 1 ml of medium 1 (a serum-free, conditioned medium prepared as described in the text); cells were not exposed to arabinosylcytosine and the medium was not renewed. In these conditions, in contrast to that observed in a, the development of glial cells was impaired. The development of neurones in the virtual absence of glial cells at day 5 is seen. $\times 187$. c, Visualization by autoradiography at day 5 of glial cell nuclei labelled by incubating mesencephalic cells for 48 h between days 3 and 5 with 9×10^{-8} M ^3H -thymidine (28 Ci mmol^{-1} , CEA) added to the culture medium. $\times 180$. After three washes with cold phosphate-buffered saline (PBS), the cells were fixed for 1 h at 4°C with 2.5% glutaraldehyde (Taab Laboratories) in PBS, rinsed four times with cold PBS, air dried and covered with Ilford K5 emulsion. Autoradiograms were developed 1 week later using Kodak Dektol. d, Visualization of labelled dopaminergic neurones at day 8 by autoradiography. $\times 183$. Cells were incubated at 37°C for 1 h with 5×10^{-7} M ^3H -DA (9.9 Ci mmol^{-1} , NEN) in 1 ml PBS complemented with 10^{-3} M magnesium, 10^{-3} M calcium and 3.3×10^{-2} M glucose. Cells were then washed and processed for autoradiography as described in c. No labelling was seen when the incubation was carried out at 0°C . Incubation at 37°C in the presence of 5×10^{-6} M desmethylinipramine (Geigy) or 10^{-6} M fluoxetine (Lilly), drugs known to inhibit ^3H -DA uptake into noradrenergic and serotonergic neurones respectively, did not modify the number of labelled cell bodies. This number was reduced to 5% of control values when the incubation was performed in the presence of 5×10^{-6} M benztropine (Merck), a potent inhibitor of ^3H -DA uptake into dopaminergic nerve cells.

substances of cellular origin or of contaminating serum components released into the medium during the 48-h conditioning period. Mesencephalic cell cultures or co-cultures with striatal cells were thus directly plated in medium 2, which consisted of a non-conditioned serum-free basal medium supplemented with hormones, proteins and salts as above (Table 1). In this medium, the development of glial cells was equally impaired (8.5 ± 3.5 , 15 ± 4.2 , $n = 3$, ^3H -thymidine-labelled cells in cultures and co-cultures, respectively). The dopaminergic neurones could be characterized as in medium 1 by autoradiography and by the estimated capacities for ^3H -DA uptake and synthesis. However, ^3H -DA uptake was lower than that observed with medium 1 and the survival of the mesencephalic neurones never exceeded 10 days. Nevertheless, the stimulatory effect of the striatal target cells on ^3H -DA uptake and synthesis was present at 8 days, as observed with medium 1 (Table 2). From these results we conclude that the use of a conditioned medium containing traces of serum components is not required for the influence of target striatal cells on dopaminergic neurones, although it favours their overall development.

A possible role of serum contaminants in the improved survival of cultures and co-cultures in medium 1 over that in medium 2 was excluded by studies with a third type of conditioned medium. Dissociated cells from the whole brain of 15-day-old mouse embryos were directly plated in the serum-free medium 2 and grown for 8 days. The medium was then removed, filtered, further complemented with proteins, hormones and salts (as described for media 1 and 2) and used as conditioned medium (medium 3, Table 1). Results comparable with those reported for experiments performed with medium 1 were obtained: absence of glial cells, satisfactory development of the neuronal populations including the dopaminergic neurones, which survived for 2 weeks at least, and facilitated maturation of the dopaminergic neurones in the presence of striatal cells, as revealed by measurement of ^3H -DA uptake at 8

and 15 days (Table 2). Thus, substances of cellular origin are apparently responsible for the longer survival of neurones in medium 1. Furthermore, these substances could originate from neurones because medium 3 was prepared in conditions impairing the development of glial cells.

Table 3 Mean value of ^3H -dopamine uptake per dopaminergic cell at 8 days in medium 1

	-STR	+STR
Expt 1		
Uptake	6,143 $\pm$ 924	9,555 $\pm$ 661*
Cell number	1,556 $\pm$ 574	1,495 $\pm$ 276
Uptake per cell	3.9	6.4
Expt 2		
Uptake	6,207 $\pm$ 523	9,134 $\pm$ 561*
Cell number	1,880 $\pm$ 416	1,207 $\pm$ 118
Uptake per cell	3.3	7.7

^3H -dopamine uptake in cultures (-STR) and co-cultures (+STR) was estimated as described in Table 2 legend (mean of four determinations). Sister cultures and co-cultures were used to count the number of labelled dopaminergic cell bodies after autoradiography as described in Fig. 1d legend. Results are the mean $\pm$ s.e.m. of triplicates. ^3H -DA uptake per cell is expressed as c.p.m. per cell.

* Difference between values obtained with cultures and co-cultures is significant ($P < 0.05$).

Due to the marked reduction of the glial cell population in the various experimental conditions used, it is unlikely that glial cells contribute to the stimulatory effect of striatal cells on the development of mesencephalic dopaminergic neurones. However, it is just possible that a minor population of striatal glial cells distinct from those evaluated by ^3H -thymidine incorporation is still present and thus involved in the phenomenon observed.

To determine whether the stimulatory effect of striatal neurones on ^3H -DA uptake and synthesis is related to a better survival or to an increased maturation of individual dopaminergic cells, the numbers of neurones labelled with ^3H -DA in cultures and co-cultures were estimated on autoradiograms. The effects of various inhibitors of amine uptake in aminergic neurones on the number of labelled cells indicated that the neurones labelled in these conditions corresponded to dopaminergic cells (Fig. 1d legend). By counting all labelled dopaminergic cell bodies, analysis of several experiments carried out with medium 1 indicated that after 8 days in culture, the total number of dopaminergic cells was not modified by the presence of striatal cells (Table 3). Similar observations were made with media 2 and 3 (data not shown). These results suggest an increased development of individual dopaminergic neurones in co-cultures and confirm previous estimations made after histochemical visualization of dopaminergic neurones grown in serum-complemented medium³.

Autoradiograms also revealed that some dopaminergic neurones were more intensely labelled than others. This may be related to the presence in the cultures of subpopulations of dopaminergic cells involved in the formation of the various ascending dopaminergic pathways. It is not known whether these subpopulations of mesencephalic dopaminergic cells respond in a similar way to the influence of striatal neurones.

We draw three main conclusions from our study: (1) the mesencephalic dopaminergic neurones could develop when cultured in serum-free medium (conditioned or not), which markedly impaired the proliferation of glial cells; (2) the dopaminergic neurones survived for a longer time when the synthetic medium was conditioned in the presence of different cell types, indicating that diffusible cellular products are required for long-term survival; (3) in the virtual absence of glial elements the striatal cells exerted a stimulatory effect on the

Table 2 Influence of striatal cells on ^3H -DA uptake or synthesis in mesencephalic cells grown in various serum-free media

	8 Days		15 Days	
	-STR	+STR	-STR	+STR
Medium 1: ^3H -DA uptake				
Expt 1:	5,882 $\pm$ 204	8,234 $\pm$ 980*	8,100 $\pm$ 904	17,820 $\pm$ 1,390*
2:	6,207 $\pm$ 523	9,134 $\pm$ 561*	9,194 $\pm$ 1,935	22,112 $\pm$ 2,002*
3:	4,449 $\pm$ 1,021	13,436 $\pm$ 2,231*	ND	ND
Medium 2: ^3H -DA uptake				
Expt 1:	2,147 $\pm$ 641	5,920 $\pm$ 135*	—	—
2:	1,422 $\pm$ 144	2,573 $\pm$ 368*	—	—
Medium 3: ^3H -DA uptake				
Expt 1:	ND	ND	3,119 $\pm$ 941	8,206 $\pm$ 646*
2:	2,286 $\pm$ 192	3,372 $\pm$ 104*	1,666 $\pm$ 484	3,620 $\pm$ 421*
Medium 1: ^3H -DA synthesis				
Expt 1:	375 $\pm$ 72	621 $\pm$ 55*	1,359 $\pm$ 58	2,605 $\pm$ 288*
2:	1,673 $\pm$ 210	2,773 $\pm$ 313*	3,198 $\pm$ 730	6,235 $\pm$ 413*
3:	497 $\pm$ 163	1,467 $\pm$ 103*	ND	ND
Medium 2: ^3H -DA synthesis				
Expt 1:	510 $\pm$ 55	846 $\pm$ 98*	—	—

Mesencephalic (13-day-old) and striatal (15-day-old) mouse embryonic cells were dissociated in basal medium as described in Fig. 1a legend, plated on non-irradiated collagen plus polyornithine and grown in 1 ml of medium 1, 2 or 3 (see Table 1 and text). Cultures (-STR) were obtained by plating 1.5×10^6 mesencephalic cells per dish; 1.5×10^6 mesencephalic cells and 1×10^6 striatal cells per dish were used in co-cultures (+STR). In several experiments ^3H -DA uptake or synthesis was estimated in 8- and/or 15-day-old cultures and co-cultures. Cells were incubated with 5×10^{-8} M ^3H -DA (9.9 Ci mmol^{-1}) for 15 min or with 1.5×10^{-6} M ^3H -tyrosine ($52.5 \text{ Ci mmol}^{-1}$, NEN) for 1 h at 37°C and then treated as described previously³ to measure their ^3H -DA content. Results expressed in c.p.m. are the mean $\pm$ s.e.m. of four determinations, the yield of the counter for tritium being 40%. ND, not determined.

* The difference between values obtained with cultures and co-cultures is significant using the Student's *t*-test ($P < 0.05$).

maturation of individual dopaminergic neurones whatever the serum-free medium used (conditioned or not). Therefore, serum-free media should facilitate the characterization of the molecules and of the neuronal interactions that intervene in the survival of mesencephalic dopaminergic neurones and in their enhanced maturation in the presence of striatal target cells.

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The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine

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Despite its very potent vasodilating action *in vivo*, acetylcholine (ACh) does not always produce relaxation of isolated preparations of blood vessels *in vitro*. For example, in the helical strip of the rabbit descending thoracic aorta, the only reported response to ACh has been graded contractions, occurring at concentrations above 0.1 μM and mediated by muscarinic receptors^{1,2}. Recently, we observed that in a ring preparation from the rabbit thoracic aorta, ACh produced marked relaxation at concentrations lower than those required to produce contraction^{3,4} (confirming an earlier report by Jelliffe⁵). In investigating this apparent discrepancy, we discovered that the loss of relaxation by ACh in the case of the strip was the result of unintentional rubbing of its intimal surface against foreign surfaces during its preparation. If care was taken to avoid rubbing of the intimal surface during preparation, the tissue, whether ring, transverse strip or helical strip, always exhibited relaxation to ACh, and the possibility was considered that rubbing of the intimal surface had removed endothelial cells⁴. We demonstrate here that relaxation of isolated preparations of rabbit thoracic aorta and other blood vessels by ACh requires the presence of endothelial cells, and that ACh, acting on muscarinic receptors of these cells, stimulates release of a substance(s) that causes relaxation of the vascular smooth muscle. We propose that this may be one of the principal mechanisms for ACh-induced vasodilation *in vivo*. Preliminary reports on some aspects of the work have been reported elsewhere^{4,6}.

Descending thoracic aortas were excised from white, male, New Zealand rabbits (2–3 kg), and trimmed free of adhering fat and connective tissue as previously described². Transverse rings of 2.5-mm width were cut with scissors or with a special cutting device containing five parallel razor blades. A transverse strip was made by cutting across a ring. Strips and rings were mounted for isometric recording of tension in oxygenated Krebs–bicarbonate solution. Throughout the preparation and mounting of rings and transverse strips, the tissue was kept wet with Krebs solution, and special care was taken to avoid unintentional rubbing of the intimal surface against a foreign surface or itself.

Figure 1a, b shows typical records illustrating both the concentration-dependent relaxation by ACh of a ring and a transverse strip of aorta precontracted with noradrenaline (NA), and the loss of the relaxing response after rubbing of the intimal surface of each preparation. If care was taken not to over-stretch the preparation during the rubbing and to allow sufficient re-equilibration time (30–60 min) after the rubbing, the procedure did not lead to any loss of sensitivity to contracting agents; indeed, apparent sensitivity to ACh as a contracting agent is increased. Rubbing also had no effect on the sensitivity of aortic preparations to a variety of nonmuscarinic relaxing agents, including glyceryl trinitrate, sodium nitrite, sodium azide, adenosine, adenylic acid and isoprenaline, or to photorelaxation⁷.

The relaxation at lower concentrations of ACh, like the contraction at higher concentrations^{1,2}, was highly sensitive to blockade by atropine. From the ratio of ACh concentrations giving equal relaxation with and without atropine (10 μM , 30 min), the dissociation constant (K_B) of the atropine–receptor complex was estimated to be 0.35 ± 0.04 nM ($n = 14$). This K_B value and the relative potencies of ACh, methacholine and carbachol (about 1, 0.3 and 0.1, respectively) in eliciting relaxation indicate that the receptor involved is of the muscarinic type. Physostigmine (0.3 μM) did not significantly increase the sensitivity to ACh.

The range of concentration over which ACh produced graded relaxation in aortic preparations precontracted with NA was usually 0.01–1 μM (Fig. 1). Occasionally, in preparations with higher than usual sensitivity to the contracting action of ACh, contraction would begin to supercede relaxation at 1 μM . Half-maximal relaxation usually occurred between 0.03 and 0.10 μM . Prolonged exposure to a fixed concentration of ACh usually resulted in some 'fade' of its relaxing effect. The degree of relaxation elicited by addition of a fixed concentration of ACh tended to decrease as the level of initial tonic contraction (tone) was increased.

ACh was about equally effective in relaxing contractions of the same magnitude produced by NA, histamine, serotonin, angiotensin or prostaglandin $F_{2\alpha}$. However, moderate to high contractions produced by increasing K^+ in the Krebs solution (by adding 12–18 mM KCl) were less sensitive to ACh, showing maximal relaxation 10–50% of that of comparable NA-induced contractions. In two experiments in which the Krebs solution bathing the aortic rings was replaced by a 'K₂SO₄–Krebs solution' (NaCl and NaHCO₃ of the regular solution substituted for by equimolar concentrations of K₂SO₄ and KHCO₃, respectively), the contractions obtained, which were ~75% of maximal NA-contractions, were relaxed about 20% by 1 μM ACh. As the K₂SO₄–Krebs solution should completely depolarize all cells of the preparations, relaxation by ACh can apparently occur independently of membrane potential changes of the smooth muscle cells.

The fact that rubbing of the intimal surface, but not the adventitial surface, removed the capacity of aortic preparations to relax in response to ACh strongly suggested that the endothelial cells were necessary for the relaxation and that they were removed by the rubbing. To investigate this possibility, the intimal surfaces of variously treated preparations were examined microscopically *en face* after application of a silver staining procedure⁸ which delineates endothelial cell borders, and also stains the subendothelial layer in areas where endothelial cells have been lost. Some typical photomicrographs are shown in Fig. 2. Unrubbed preparations in which ACh produced 80–100% relaxation of moderate tone had usually retained 60–75% of the endothelial cells by the end of an experiment. In 'inadequately' rubbed preparations it was not unusual to observe about 20% relaxation of moderate tone when only a few per cent of the endothelial cells were retained. When rubbing resulted in the complete loss of the relaxing response to ACh, there was always virtually a complete loss of endothelial cells. Thus, the staining procedure clearly showed that endothelial cells were required for relaxation by ACh. Preparations were

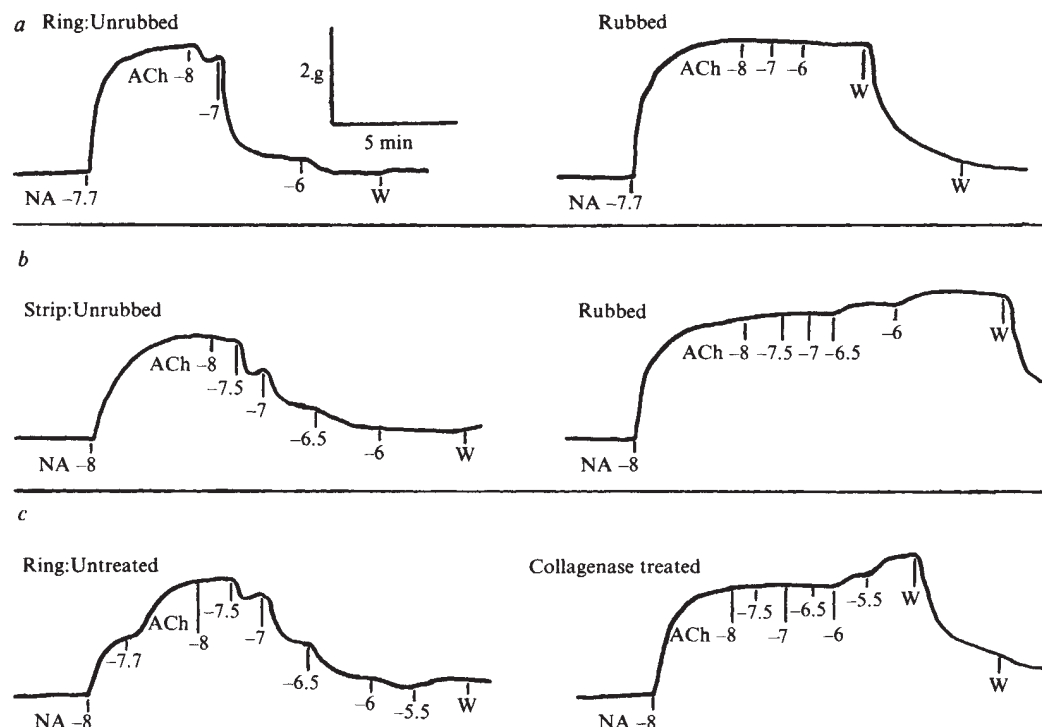


Fig. 1 Loss of relaxing response of preparations of rabbit aorta to ACh after rubbing of the intimal surface or exposure to collagenase. Rings and transverse strips were mounted in all-glass 20-ml muscle chambers at 37 °C. The bathing fluid was Krebs solution of the following composition (mM): NaCl 118, KCl 4.8, CaCl₂ 2.5, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 24, glucose 11 and Na₂-EDTA 0.03. It was continuously gassed with 95% O₂-5% CO₂. Two hooks of stainless steel wire (0.6-mm diameter), bent in a modified L-shape, were used to mount each ring. The short straight portion of each hook passed through the lumen of the ring. The lower hook was attached to the base of the muscle chamber, the upper to a strain gauge. For mounting each strip, two small lucite clips with jaws under spring tension were used. Hooks connected the lower clip to the base of the muscle chamber, and the upper clip to a strain gauge. All preparations were first equilibrated for at least 2 h, and basal tension was set to 2 g before drug additions. The records show the point at which each drug addition was made, and the corresponding cumulative concentration of the drug in the chamber after the addition, expressed as the log of the molar concentration. NA indicates noradrenaline; W indicates washout of the chamber. *a*, Recordings from the same ring before and after rubbing of its intimal surface. After the record on the left had been obtained, the ring was removed from the chamber on its mounting hooks. While it was kept under tension and moist with Krebs solution, its intimal surface was gently rubbed for 1 min with a small, wooden applicator stick that had been shaved down to permit easy insertion through the lumen. The ring was then resuspended in the muscle chamber and retested about 30 min later. A matched ring that was rubbed for the same time on its adventitial surface exhibited no loss of its capacity for relaxation by ACh (not shown). *b*, Simultaneous recordings from an unrubbed (left) and rubbed (right) transverse strip from the same aorta. Before being placed in clips for mounting, the latter strip had been dragged slowly for 1 min with intimal surface down, over a sheet of filter paper wetted with Krebs solution. A third strip, that had been similarly dragged with adventitial surface down, exhibited no loss of relaxation capacity (not shown). *c*, Simultaneous recordings from rings made from the same aorta before and after exposure of the intimal surface to a solution of collagenase, similar to that used by Jaffe *et al.*⁹, and containing 0.2% of the CLS-type enzyme preparation (Worthington) in a phosphate-buffered saline solution (PBS). The control ring (left) was cut first and incubated for 25 min in PBS alone at 37 °C. The remaining segment of aorta was cannulated at its upper end and closed with a clip at its lower end. It was then filled with collagenase solution and immediately immersed in PBS at 37 °C. After the desired exposure time, a ring was cut from near the lower end of the segment. The record on the right is for a ring whose intimal surface had been exposed to the collagenase solution at 37 °C for 15 min. Similar results were obtained in three other experiments. Rings whose intimal surfaces had been exposed for only 7 min showed about a 50% reduction in maximal relaxing response to ACh (not shown). A 15-min exposure to collagenase did not interfere with contraction elicited by high concentrations of ACh nor with relaxation by other agents such as isoprenaline, sodium nitrate and 5'-AMP.

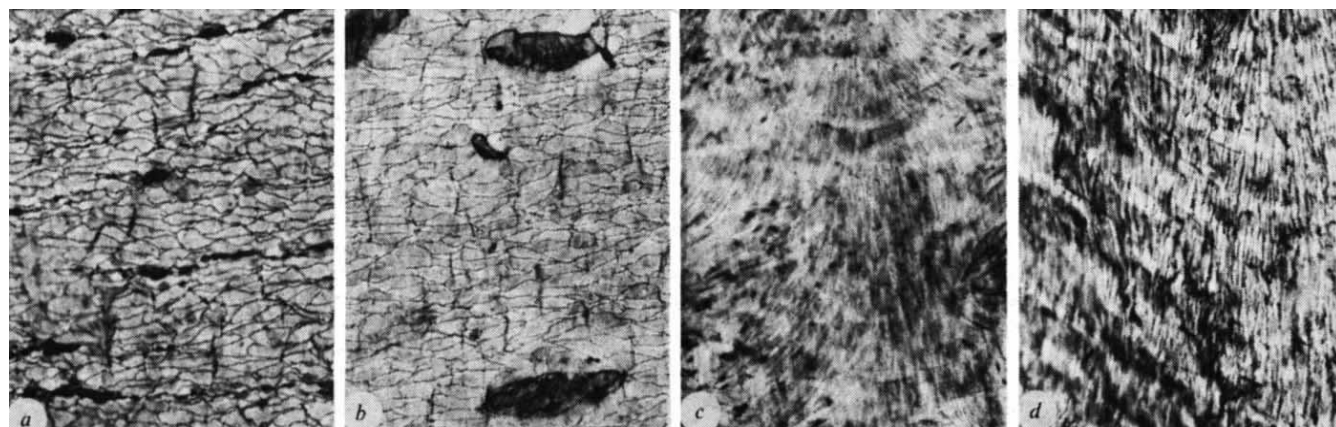


Fig. 2 Photomicrographs of the intimal surface of aortic preparations after various treatments. A preparation, cut open in strip form, was laid adventitia-side down on a small piece of paper towel, to which it adhered well during exposure to each solution in the silver staining procedure. The procedure was that of Poole *et al.*⁸, with the modification that the blood vessel preparation moved about in each solution for the recommended time, while on the paper-towel backing. After final fixation in 5% formalin, the preparation could be examined microscopically *en face* in the wet state, or it could be dehydrated and imbedded in a permanent mount. The photomicrographs here are of preparations after imbedding. Magnification is $\times 218$. *a*, Strip that was stained immediately after being cut from freshly excised and trimmed aorta. Almost all the intimal surface was covered by endothelial cells. *b*, Strip made from an unrubbed ring at the end of an experiment on drug testing. During the experiment ACh at 10^{-6} M relaxed moderate NA-induced tone in this ring by about 95%. Only about 70% of the intimal surface (excluding those parts that had been apposed to the surface of the metal mounting hooks) was covered by endothelial cells. It was rare to find more than 80% retention of endothelial cells in unrubbed preparations examined at the end of an experiment. *c*, Strip that had had its intimal surface rubbed on filter paper for 1 min before being used in an experiment in which it gave no relaxation in response to ACh (similar to Fig. 1b). The surface was devoid of endothelial cells. *d*, Strip made from a ring from a segment of aorta that had been exposed intraluminally to 0.2% collagenase for 15 min at 37 °C. During the experiment the ring gave no relaxation in response to ACh (similar to Fig. 1c). No endothelial cells were left on the intimal surface.

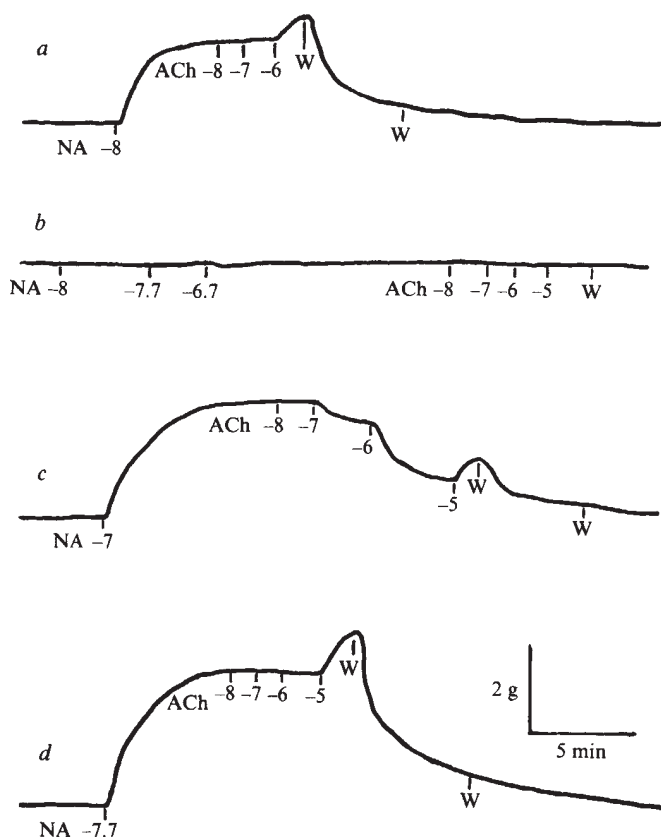


Fig. 3 Demonstration of release of a relaxing substance (or substances) from endothelial cells by ACh. Rings of uniform width (2.5 mm) were cut from a segment of descending thoracic aorta, and then cut across to yield transverse strips. Before the strips were mounted in muscle chambers, the intimal surfaces of three of them were rubbed on filter paper (as in Fig. 2b) to remove their endothelial cells. A second segment of aorta was incubated without tension in Krebs solution at 37 °C, and from it longitudinal strips (strips running parallel to the long axis) were cut as needed in the same dimensions as the transverse strips. Initially, all strips mounted for recording were mounted singly with plastic clips. Later, certain pairs of strips were mounted together in sandwich form, with their entire intimal surfaces apposed. *a*, NA and ACh on a transverse strip with endothelium removed. ACh did not relax NA-induced tone, and evoked additional contraction at 1 μ M. *b*, NA and ACh on a longitudinal strip with endothelium present. Only trivial changes in tension were exerted on the transducer (because of the orientation of the smooth muscle cells). *c*, NA and ACh on a sandwich made from the transverse strip without endothelium (same strip as in *a*) and a longitudinal strip with endothelium. The latter strip was cut from the incubated but unmounted segment of aorta in a region adjacent to that from which the strip that gave record *b* was cut. The sandwich was allowed to equilibrate for about 30 min after mounting. Note the relaxation at 10^{-7} and 10^{-6} M ACh. This was relaxation of the transverse strip, as it was this strip in the sandwich that gave the recorded tension increase on addition of NA. *d*, Test of NA and ACh on the transverse strip without endothelium after removing the longitudinal strip with endothelium. As in *a*, it now again gave no relaxation in response to ACh. In this experiment relaxation by ACh was also demonstrated in a second transverse strip without endothelium only when it was mounted in a sandwich preparation with a longitudinal strip with endothelium. However, in a sandwich preparation made with a longitudinal strip that had also been freed of endothelium (by rubbing) and a transverse strip without endothelium, the latter failed to relax in response to ACh. The results in this experiment were replicated in four similar experiments.

also studied in which endothelial cells had been removed by exposing the intimal surface to commercial collagenase⁹. Figure 1c shows that a ring from a collagenase-treated segment failed to relax in response to ACh, and Fig. 2d shows that the intimal surface of such a ring was completely free of endothelial cells.

To explain the obligatory role of endothelial cells in the relaxation, it was postulated that ACh acting on a muscarinic receptor in these cells stimulates them to release a substance (or substances) which in turn acts on the smooth muscle cells in the media to activate relaxation. Direct evidence for this hypothesis

was obtained in experiments in which a regular transverse strip freed of endothelial cells was tested when mounted separately and also when mounted, intimal surface against intimal surface, with a longitudinal strip of the same width and length with endothelial cells present (Fig. 3). Because of the orientation of its muscle cells, the longitudinal strip could contribute only trivial increases in tension on the strain gauge during contraction. As shown in Fig. 3, ACh, which failed to relax contraction of the transverse strip mounted separately, gave good relaxation of contraction of the same strip when it was mounted as a 'sandwich' with the longitudinal strip.

Although we have not identified the released relaxing substance(s), we have ruled out bradykinin, prostacyclin, cyclic AMP and cyclic GMP (no relaxation or only slight relaxation of

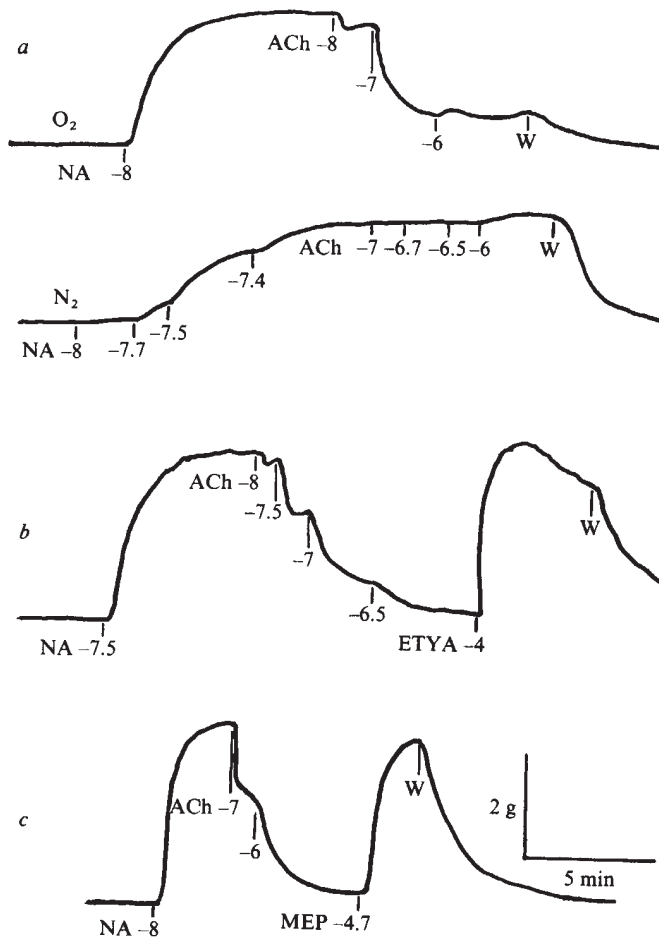


Fig. 4 Inhibition of the relaxing action of ACh by anoxia, ETYA and mepacrine (MEP). *a*, Response of an aortic ring to ACh under oxygen (upper record) and under anoxia (lower record). About 30 min before making the lower record, 95% N₂-5% CO₂ was substituted for 95% O₂-5% CO₂, and was bubbled rapidly through the solution in the muscle chamber. To minimize the chance of significant traces of O₂ from air getting into the solution, the top of the chamber was covered with a plastic cap containing only two small holes, one for passage of the thread to the transducer and the other for introduction of needles of Hamilton syringes in adding drugs. In this and three similar experiments, the relaxing response to ACh was completely lost under anoxia. It was completely restored on return of the preparation to O₂ (not shown). *b*, Acute inhibition by ETYA of ACh-induced relaxation. The ETYA was added as a solution in ethanol in a volume of 60 μ l to the 20 ml of Krebs solution in the muscle chamber. The addition of 60 μ l of ethanol alone in control experiments gave only small variable increases in tone (not shown). The ETYA concentration of 10^{-4} M represents the concentration that would have been obtained if all ETYA had stayed in solution in the muscle chamber; however, some always came out of solution. The fall in tone that began about 2 min after the addition of ETYA and before washout (W) is the result of a marked (but reversible) depression of responsiveness to contractile agents that develops in aortic preparations on exposure to ETYA. *c*, Acute inhibition by mepacrine of ACh-induced relaxation. Mepacrine inhibition was readily reversed by washout.

aortic preparations), and also adenosine and 5'-AMP (maximal relaxation much less than with ACh in some experiments). In addition to prostacyclin, other products of cyclo-oxygenase activity were ruled out because inhibitors of that enzyme, indomethacin (40 μ M) and aspirin (100 μ M), had no effect on the relaxing action of ACh.

When aortic preparations were made anoxic, ACh produced very little or no relaxation (Fig. 4a). In the same conditions, agents like sodium nitrite, glyceryl trinitrate and isoprenaline still produced good relaxation. Inhibition of ACh-induced relaxation by anoxia has been reported previously for rings of dog femoral artery¹⁰. Furthermore, when 5, 8, 11, 14-eicosatetraenoic acid (ETYA) was added in sufficient concentration, it rapidly (<1 min) and often completely antagonized an ACh-induced relaxation (Fig. 4b). Moreover, exposure of preparations to 100 μ M ETYA for 30–60 min completely and irreversibly inhibited relaxation by ACh. Mepacrine at 20 μ M almost completely inhibited relaxation by ACh, whether it was added before or after the ACh. As ETYA is an inhibitor of lipoyxygenase and cyclo-oxygenase¹¹ and as mepacrine inhibits the release of arachidonic acid from certain phosphatides (possibly by inhibiting phospholipase A₂ (ref. 12), these results suggest that ACh acting on the muscarinic receptor of the endothelial cells somehow activates a reaction sequence in which arachidonic (or some other unsaturated fatty acid) is liberated and then oxidized by lipoyxygenase to a product that is responsible for the relaxation of the smooth muscle cells. The involvement of lipoyxygenase is attractive despite negative results with the lipoyxygenase inhibitor BW755C (ref. 13).

In experiments on other arteries, we have found that rings from the following exhibit ACh-induced relaxation before, but not after, rubbing of the intimal surface: rabbit superior mesenteric, pulmonary and ear arteries; rat thoracic aorta; guinea pig thoracic aorta, cat thoracic and abdominal aortae, superior mesenteric, pulmonary and external iliac arteries, dog circumflex and left anterior descending coronary arteries.

In relating the present *in vitro* findings to the vasodilating effect of ACh *in vivo*, it should be remembered that vasoconstriction of isolated blood vessels produced by stimulation of adrenergic nerves can be antagonized by ACh acting on pre-junctional muscarinic receptors of the nerve terminals^{14–17}. As this antagonism depends on inhibition of the stimulation-evoked release of the adrenergic neurotransmitter NA^{16–18}, it is a mechanism for vasodilatation by ACh only in blood vessels that are vasoconstricted by nerve stimulation. The endothelium-requiring mechanism explored here would be effective whether or not the vasoconstriction was caused by nerve stimulation. We propose that this mechanism applies in the case of smaller resistance vessels as well as for the larger arteries used in this study, and that it is one of the principal mechanisms responsible for the very potent vasodilating effect of ACh and other muscarinic agonists in intact animals.

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Cholecystokinin octapeptide in the rat hypothalamo-neurohypophyseal system

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Gastrin-cholecystokinin (CCK)-like immunoreactivity has been visualized by immunohistochemistry in the vasopressin-oxytocin neurosecretory system comprising the posterior lobe of the pituitary, the supraoptic and paraventricular nuclei of the hypothalamus¹. This CCK-like substance has not been chemically identified in the rat, but has been reported to be gastrin 17 in the swine pituitary². We report here that this CCK-like immunoreactive substance co-chromatographs with CCK8 sulphate on Sephadex G-50 and two HPLC chromatographic systems. No gastrin 17 was detected in rat pituitary or brain. We further report that about 60% of the CCK in posterior lobe originates in cell bodies in the paraventricular nucleus of the hypothalamus. The CCK content of posterior pituitary is dramatically decreased by physiological perturbations which stimulate vasopressin or oxytocin release. We propose that CCK may either be co-secreted with vasopressin and oxytocin to act on peripheral targets or may be involved in the regulation of vasopressin or oxytocin neurosecretion.

The CCK-like substance extracted with 90% methanol from rat posterior pituitary glands was fractionated on three chromatographic columns which separate CCK8 from gastrin: a Sephadex G-50 column, a HPLC reverse-phase C-18 column, and a HPLC molecular-sieving column. The pattern of elution of pituitary CCK immunoreactive material from these chromatographic columns was measured with a radioimmunoassay (RIA) which displayed 70% cross-reactivity with gastrin.

When a rat posterior pituitary extract was chromatographed on Sephadex G-50 the immunoreactivity emerged in one peak that had the same elution volume as synthetic sulphated CCK8 (Fig. 1a). Sephadex chromatography coupled with RIA has been used in the past to separate the molecular forms of CCK in central and peripheral nerves³ and was, therefore, the first method applied in this study. However, as HPLC offers better

Table 1 CCK content of posterior lobe in control and paraventricular lesioned rats

	CCK (pg per lobe)
Control (6)	219 ± 46
Paraventricular (6) nucleus lesioned	89 ± 40

Bilateral paraventricular nucleus lesions were made under ether anaesthesia. Animals were placed in a Kopf stereotaxic device, 5° nose down. The electrode tips were positioned 6.4 mm rostral to the interauricular line, 0.4 mm lateral to the midline, 7.5 mm ventral to the dural surface. A current of 3 mA was passed through each electrode for 30 s. The lesions destroyed both paraventricular nuclei and damaged adjacent structures (anterior hypothalamic nuclei, ventral thalamus, perifornical area) to varying degrees. One week after the paraventricular nucleus lesions, the posterior lobe CCK content of lesioned Osborne-Mendel female rats and age-matched controls was determined. Note that strain differences exist in posterior pituitary CCK levels. Zivic-Miller rats have higher levels than Osborne-Mendel animals. Values are given as mean ± s.e.m.

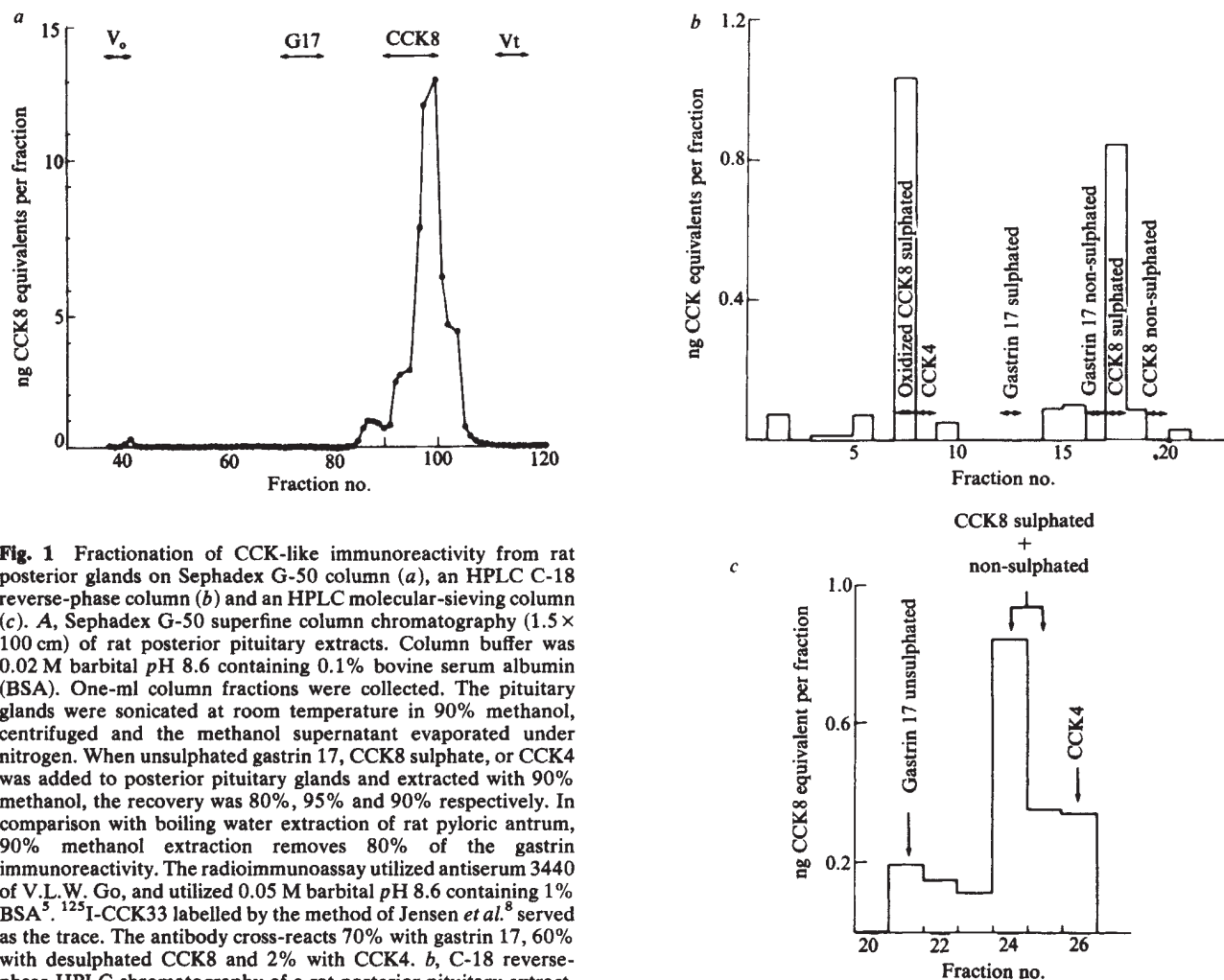


Fig. 1 Fractionation of CCK-like immunoreactivity from rat posterior glands on Sephadex G-50 column (a), an HPLC C-18 reverse-phase column (b) and an HPLC molecular-sieving column (c). **a**, Sephadex G-50 superfine column chromatography (1.5 × 100 cm) of rat posterior pituitary extracts. Column buffer was 0.02 M barbital pH 8.6 containing 0.1% bovine serum albumin (BSA). One-ml column fractions were collected. The pituitary glands were sonicated at room temperature in 90% methanol, centrifuged and the methanol supernatant evaporated under nitrogen. When unsulphated gastrin 17, CCK8 sulphate, or CCK4 was added to posterior pituitary glands and extracted with 90% methanol, the recovery was 80%, 95% and 90% respectively. In comparison with boiling water extraction of rat pyloric antrum, 90% methanol extraction removes 80% of the gastrin immunoreactivity. The radioimmunoassay utilized antiserum 3440 of V.L.W. Go, and utilized 0.05 M barbital pH 8.6 containing 1% BSA³. ¹²⁵I-CCK33 labelled by the method of Jensen *et al.*⁸ served as the trace. The antibody cross-reacts 70% with gastrin 17, 60% with desulphated CCK8 and 2% with CCK4. **b**, C-18 reverse-phase HPLC chromatography of a rat posterior pituitary extract. Chromatography was through a Supelco LC-18 HPLC column⁹. Elution buffer was 22% acetonitrile-78% triethylamine-phosphoric acid buffer pH 6.5. The position of partially and completely oxidized CCK and gastrin was determined by deliberately oxidizing the peptide with hydrogen peroxide and following the time course of the oxidation with the HPLC. **c**, Varian 2000 molecular-sieving HPLC column chromatography⁹ of a rat posterior pituitary extract. Elution buffer was phosphate-buffered saline (PBS) pH 6.5. Before radioimmunoassay the fractions were passed through and eluted from a Waters C-18 Sep-pack to remove the PBS.

resolution of CCK peptides than Sephadex, two HPLC systems were also utilized to chromatograph rat posterior pituitary extracts.

In Fig. 1b the elution of a rat posterior pituitary extract from a HPLC reverse-phase C18 column is shown. All of the immunoreactivity emerges in two column fractions, one of which coincides with partially oxidized sulphated CCK8 and the other with reduced (that is, native) sulphated CCK8. No immunoreactivity co-migrates with sulphated or unsulphated gastrin 17 (in either their native or oxidized forms) or with CCK4 (Trp-Met-Asp-Phe-NH₂). However, although 90% methanol can efficiently extract CCK4, the cross-reactivity of the antibody with CCK4 is sufficiently low that the existence of some CCK4 cannot be excluded.

In Fig. 1c the elution pattern of a rat pituitary extract from an HPLC molecular-sieving column is shown. The bulk of the immunoreactivity elutes with sulphated and desulphated CCK8; little material elutes with gastrin 17. Thus, sulphated CCK8 is the dominant CCK-like immunoreactive substance in the posterior pituitary of the rat, as it is in rat brain³⁻⁵. The presence of large amounts of gastrin 17 in the pig pituitary² may reflect species differences.

The distribution of CCK in the hypothalamus and pituitary resembles the distributions of vasopressin and oxytocin. As mentioned above, CCK-containing cell bodies have been visualized in the supraoptic and paraventricular nuclei by means of immunocytochemistry, and a dense collection of CCK fibres is

found in the median eminence and posterior pituitary^{1,6}. Considerable CCK was detected by RIA in Osborne-Mendel female rats in paraventricular and supraoptic nuclei (0.86 ± 0.1 and 1.5 ± 0.4 ng CCK8 equivalents per mg protein ($n=3$), respectively), more CCK in median eminence (2.3 ± 0.5 ng per mg protein) and still more in posterior pituitary (4.4 ± 1.3 ng per mg protein or 200–300 pg per lobe). CCK was undetectable (<10.0 pg per pituitary) in the intermediate and anterior lobes of the pituitary.

To determine whether the CCK in the posterior pituitary is intrinsic to it, the CCK content was measured in pituitaries of stalk sectioned Zivic-Miller rats. One week after the stalk sections were performed the CCK content of the posterior pituitaries was undetectable. This indicates that all of the CCK in the posterior pituitary has an extrapituitary origin. The paraventricular nucleus contributes a substantial portion of the posterior lobe's CCK as paraventricular nucleus lesions reduced the CCK content of the posterior pituitary by 60% (Table 1).

To determine whether CCK might be secreted in parallel with vasopressin and/or oxytocin, the posterior pituitary CCK content was determined in lactating rats and rats given 2% saline to drink. The results are shown in Table 2. In lactating animals whose oxytocin release is increased, the CCK content in the posterior pituitary was reduced to 17% of control. In rats given 2% saline to drink for 5 days, a treatment which is known to reduce vasopressin by 80% (ref. 7), the pituitary CCK content was about 20% of control.

Table 2 CCK in posterior lobe in lactating and salt-treated rats

	CCK (pg per lobe)
Controls (9)	257 ± 36
Salt treated (6)	56 ± 20
Lactating (3)	43 ± 4

Osborne-Mendel female rats (250 g) which were given 2% NaCl for 5 days were compared with controls given tap water. Lactating Osborne-Mendel rats were separated from their pups and compared with age-matched female controls. The CCK concentrations of both control groups were the same so the data were pooled. Numbers in parentheses refer to the number of animals tested. This experiment was repeated once with similar results. Values are mean ± s.e.m.

The CCK content of posterior pituitary is thus dramatically decreased by physiological perturbations which stimulate the release of vasopressin and oxytocin. We propose that CCK may either be co-secreted with vasopressin and oxytocin to act on peripheral targets, or may be involved at the pituitary level in the regulation of vasopressin or oxytocin secretion. Whether CCK is found in the same or different neurones as vasopressin or oxytocin remains to be determined.

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VIP as a possible neurotransmitter of non-cholinergic non-adrenergic inhibitory neurones

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Many peripheral autonomic nerves are neither cholinergic nor adrenergic. Such nerves are widely distributed in the gastrointestinal, urogenital and respiratory tracts, and in blood vessels^{1,2}. The nature of their neurotransmitter is not known. We have previously reported that vasoactive intestinal polypeptide (VIP) is a potent inhibitor of opossum lower oesophageal sphincter (LOS) and that its inhibitory effect is exerted directly on the sphincter muscle³. Subsequent studies have confirmed the inhibitory effect of VIP on LOS in other species^{4–6}. Recently, very high tissue levels of VIP have been reported in the LOS and other gastrointestinal sphincters⁷. Furthermore, VIP has been localized to intramural neurones and is released upon electrical stimulation of the vagus nerve^{8,9}. We report here that immunoantagonism of VIP with a high-titre antiserum antagonized inhibitory neuromuscular transmission in the LOS. These findings provide evidence of a role for VIP as an inhibitory neurotransmitter.

Experiments were performed in opossums (*Didelphis virginiana*) anaesthetized with pentobarbital (40 mg per kg). Opossum LOS is suitable for these studies because it maintains a resting tone independent of tonic neural activity and the vagus nerves carry predominantly inhibitory influences to the LOS¹⁰. Moreover, the inhibitory neurones that lie in the vagal pathway have been shown to be non-cholinergic and non-adrenergic in nature¹¹.

The lower oesophageal sphincter pressures (LOSP) were monitored with water-filled, continuously perfused catheters that were anchored in the LOS as described elsewhere¹². Ringer's solution, control serum (non-immune serum from an unimmunized rabbit), VIP antiserum or VIP antiserum preabsorbed *in vitro* with native VIP, were infused into the branch of left gastric artery that supplies the sphincter¹³. VIP antiserum no. 89N was raised in a rabbit immunized with a conjugate of natural porcine VIP and bovine serum albumin. Using the antiserum in a dilution of 1:300,000 at 4 °C, the equilibrium constants of association were found to be $K_{a1} = 6.7 \times 10^{10} \text{ M}^{-1}$ and $K_{a2} = 2.3 \times 10^9 \text{ M}^{-1}$. The binding capacity of the undiluted antiserum was calculated to be 57 µg VIP per ml of antiserum. *In vitro* assay showed no cross-reactivity with gastric inhibitory polypeptide, glucagon, secretin, substance P, porcine pancreatic polypeptide, cholecystokinin or gastrin. The antiserum from the same batch was neutralized by addition of VIP (57 µg VIP per ml of antiserum). The mixture was kept overnight and then centrifuged to remove any precipitate before its use.

The brachial vein was used for intravenous (i.v.) administration of VIP. Vagal efferent stimulation was provided by electrical stimulation of the peripheral end of the cut vagus nerve in the neck¹⁰. Stimulation of intramural neurones was achieved with electrodes inserted into the wall of the sphincter as described elsewhere¹². Nicotine (40 µg per kg) was administered intravenously¹⁴.

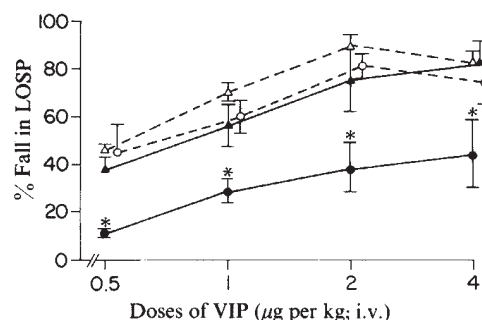


Fig. 1 The influence of intra-arterial VIP antiserum on the inhibitory effect of intravenously administered VIP on the LOSP. The corresponding Ringer controls for control serum (○, 1:12.5) and VIP antiserum (●, 1:12.5) are represented by △ and ▲ respectively. VIP antiserum significantly (*) antagonized the effect of all doses of VIP studied. This antagonism was significant ($P < 0.05$) when compared to either control serum or the Ringer's infusion. Each point is mean ± s.e. of four observations.

Two separate groups, each of four animals, were studied. One group received an infusion of Ringer's solution followed by control serum. The second group received Ringer's solution followed by VIP antiserum. All infusions were made at the rate of 5 ml h⁻¹. The pressure recording was coded and results were analysed blindly. Intravenous administration of VIP produced a dose-dependent fall in LOSP, which was not modified by control serum (1:12.5 dilution). In contrast, VIP antiserum (1:12.5) significantly ($P < 0.05$) antagonized the inhibitory effect of VIP at all dose levels (Fig. 1). Thus the antiserum was effective in partially antagonizing the effect of exogenous VIP. Higher dilutions of antiserum 1:50 were ineffective and the dilution of 1:25 produced inconsistent effects.

Electrical stimulation of vagal efferents produced a frequency-dependent fall in the sphincter pressure. Other

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parameters of stimulation were 10 V, 0.5 ms square pulses for 2-s train duration. Infusion of control serum did not modify vagal-stimulated sphincter relaxation at any of the frequencies of stimulation examined ($P > 0.05$, Fig. 2).

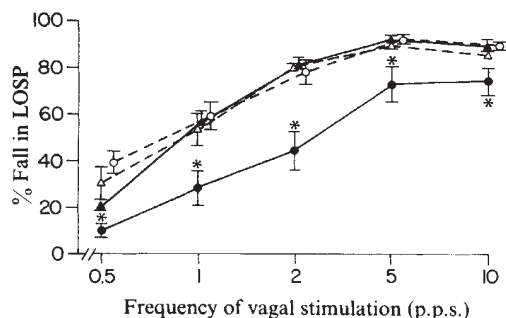


Fig. 2 The influence of VIP antiserum on the vagal-stimulated fall in LOSP. Note that vagal stimulation produced frequency-dependent fall in LOSP. VIP antiserum significantly (*) antagonized the inhibitory effect of vagal stimulation at all the frequency levels studied ($P < 0.05$). Control serum did not modify the effect of vagal stimulation. The corresponding Ringer controls in two different groups for control (○) and VIP antiserum (●) are represented by Δ and ▲ respectively. Each point is mean $\pm$ s.e. of 9–12 observations. p.p.s., Pulses per s.

As the vagal pathway to the LOS muscle consists of preganglionic fibres that synapse with postganglionic inhibitory neurones¹¹, the effect of VIP antiserum on vagal-stimulated sphincter relaxation could be exerted at the preganglionic or at the postganglionic level. To examine these alternatives we investigated the effect of postganglionic neuronal activation with intramural electrical stimulation. We have previously shown that intramural stimulation in this preparation activates postganglionic neurones as the effect of such a stimulation is not antagonized by synaptic block with a combination of hexamethonium and atrophine¹⁵. Effect of ganglionic stimulant nicotine¹⁴ was also examined.

LOS relaxation induced by intramural stimulation was significantly antagonized by VIP antiserum but was unaffected by control serum (Fig. 3). Likewise, the fall in LOSP induced by nicotine was antagonized by VIP antiserum but not by control serum. Nicotine (40 μ g per kg) caused 83.4 ± 3.2 (s.e.) per cent fall in LOSP during control serum and 58.7 ± 5.9 per cent during VIP antiserum infusion ($P < 0.05$, $n = 5$). These results suggest that the VIP antiserum acted at the postganglionic neuromuscular site.

The effect of VIP antiserum on all the stimuli studied above could theoretically be due to its non-selective action on the LOS

smooth muscle. To examine this, we studied the effect of a direct-acting inhibitory agent, isoproterenol. VIP antiserum did not modify its inhibitory action. Isoproterenol (2.5 μ g per kg) caused 84.7 ± 3.1 per cent fall in LOSP during the infusion of control serum and 85.0 ± 2.6 per cent during the VIP antiserum infusion ($P > 0.05$, $n = 5$).

The control serum used in these studies was not obtained from the same animal that provided the VIP antiserum. Theoretically the observed influence of the VIP antiserum could have been due to an unknown inhibitor other than the VIP antibody. To test this, we examined the influence of VIP antiserum that was preabsorbed with VIP before administration. As summarized in Table 1, preabsorbed VIP antiserum did not modify the effects of exogenous VIP, vagal or transmural stimulation in the same animal. These observations support the view that the inhibitory effect of VIP antiserum was due to the VIP antibody.

These immunopharmacological studies provide evidence for the role of VIP as a neurotransmitter of the inhibitory neurones in the LOS. This conclusion is supported by immunohistochemical demonstration of VIP in the intramural neurones and its release upon electrical stimulation of vagus nerves^{9,16–19}.

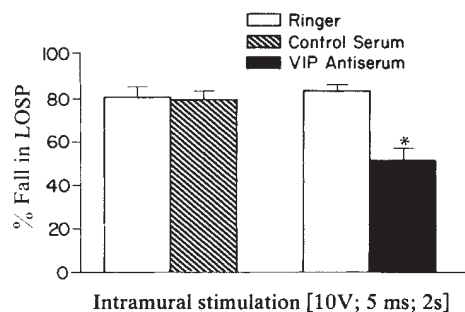


Fig. 3 Influence of VIP antiserum on the effect of intramural stimulation on LOSP. Note that VIP antiserum significantly (*) antagonized the inhibitory effect of intramural stimulation ($P < 0.05$). Control serum, however, failed to modify the effect of intramural stimulation. Each point is mean $\pm$ s.e. of 12 observations.

The VIP antiserum in the dilution of 1:12.5 did not fully antagonize the effect of exogenous VIP or of inhibitory neurone stimulation. This may be because the large size of the antibody makes it difficult for the antibody molecule to achieve sufficiently high concentrations in the extracellular space at the site of neuromuscular transmission. Moreover, the time course and the affinity of binding of the released VIP with the antiserum compared to that with the muscle receptors may also influence the degree of the antagonism. Higher concentration of antiserum could further antagonize the inhibitory response to exogenous or neurally-released VIP. Further studies are needed to resolve these issues. Alternatively, VIP may be only one of two or more neurotransmitters that are released by non-adrenergic, non-cholinergic inhibitory neurones²⁰. Burnstock originally suggested that ATP or a related nucleotide is the neurotransmitter of the non-cholinergic, non-adrenergic neurones, which he termed 'purinergic' neurones¹. Recent studies, however, do not support the view of purinergic nature of the inhibitory neurotransmitter in the opossum LOS²¹.

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Table 1 Influence of preabsorbed VIP antiserum on the effect of exogenous VIP or neurally stimulated LOS relaxation

Per cent fall in LOSP				
	No. of observations	Ringer's solution (Mean $\pm$ s.e.)	Preabsorbed VIP antiserum (mean $\pm$ s.e.)	<i>P</i> value
VIP (4 μ g per kg; i.v.)	3	72.3 $\pm$ 3.5	75.0 $\pm$ 2.9	>0.05
Vagal stimulation				
0.5 Hz	5	23.8 $\pm$ 4.4	20.8 $\pm$ 4.8	>0.05
1 Hz	5	45.8 $\pm$ 3.1	42.2 $\pm$ 7.5	>0.05
2 Hz	5	61.7 $\pm$ 7.5	58.2 $\pm$ 3.1	>0.05
5 Hz	5	73.6 $\pm$ 3.5	81.5 $\pm$ 5.0	>0.05
10 Hz	5	69.5 $\pm$ 7.9	75.7 $\pm$ 7.2	>0.05
Intramural stimulation	5	75.3 $\pm$ 4.9	82.8 $\pm$ 4.6	>0.05

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Peptidergic transmitters in synaptic boutons of sympathetic ganglia

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In sympathetic ganglia of the bullfrog, a slow synaptic potential lasting for minutes—the late slow excitatory postsynaptic potential (e.p.s.p.)—was discovered¹. This slow response, unlike other previously known synaptic potentials in the autonomic nervous system, is not mediated by acetylcholine or monoamines. Similar non-cholinergic, non-adrenergic slow synaptic potentials have since been found in several other vertebrate autonomic ganglia². We found that the late slow e.p.s.p. is probably mediated by a peptide that is identical to, or closely resembles, mammalian luteinizing hormone releasing hormone (LHRH)³, because (1) when applied directly to sympathetic neurones, LHRH and its agonists elicit a slow depolarization, associated with similar changes in membrane conductance and excitability as those occurring during the late slow e.p.s.p. Furthermore, both peptide-induced and nerve-evoked responses are blocked by antagonists of LHRH; and (2) radioimmunoassays indicate that a chain of sympathetic ganglia contains 100–800 pg of a LHRH-like peptide. Its distribution among spinal nerves, the great reduction of this substance following denervation, and its release from ganglia following isotonic KCl treatment or nerve stimulation suggest that the LHRH-like material is contained in preganglionic nerve fibres. Here we report that immunohistochemical staining of sympathetic ganglia shows that LHRH-like immunoreactivity is indeed present in synaptic boutons. We also show that the two types of ganglion cells (B cells and C cells) receive strikingly different patterns of peptidergic innervation.

The ninth and tenth ganglia of the bullfrog, the two most caudal ones in the paravertebral sympathetic chain, were used in these experiments because extensive electrophysiological studies have used them. By using several anti-LHRH sera raised in different laboratories, and the peroxidase–antiperoxidase (PAP) technique of Sternberger and co-workers⁴, we stained many boutons on the ganglion cell soma, particularly around the axon hillock region (Fig. 1a). To ensure that the staining was specific for LHRH-like immunoreactivity, we used the same anti-LHRH sera preadsorbed with LHRH in control experiments. Figure 1b shows that preadsorption completely abolishes the staining. Thus, the immunohistochemical procedure shows that the LHRH-like immunoreactivity is confined to synaptic boutons in sympathetic ganglia.

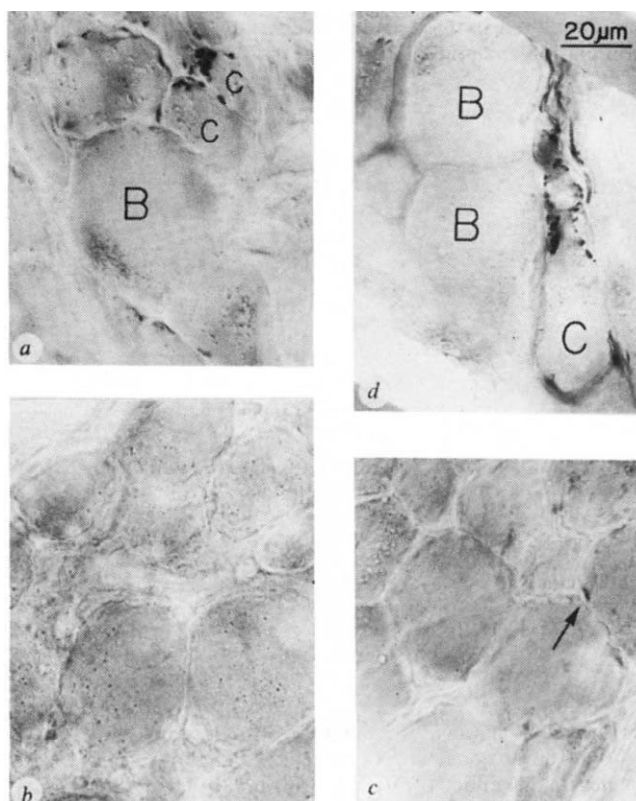


Fig. 1 Vibratome-sectioned sympathetic ganglia treated with *a*, anti-LHRH sera, or *b*, same anti-LHRH sera preadsorbed with excess synthetic LHRH ($40 \mu\text{g ml}^{-1}$), according to the PAP procedure of Sternberger⁴. In *c*, the seventh and eighth rami had been cut 5 days before the ganglia were stained with anti-LHRH sera (arrow indicates residual staining). In all cases, bullfrogs were anaesthetized with ethyl *m*-aminobenzoate and perfused through the ventricle with 4% paraformaldehyde, 0.1% glutaraldehyde in 0.15 M phosphate buffer at pH 7.4. The sympathetic chains were removed, postfixed in the same fixative for 30 min, and cut into 50- μm sections on a vibratome. Rabbit antisera to LHRH were diluted 1:1,000 with 0.05 M Tris-buffered saline, pH 7.6, containing 1 mg ml^{-1} bovine serum albumin, 1% normal sheep serum, and 0.4% Triton X-100 and used to treat sections of ganglia for 24 h at 4°C. This incubation with primary rabbit antisera was followed sequentially by an incubation with sheep antisera specific for rabbit gamma immunoglobulins (IgG) and an incubation with rabbit PAP complex. Sheep antisera against rabbit IgG and rabbit PAP were used at 1:20 dilution in Tris-buffered saline containing 1% normal sheep serum and 0.02% Triton X-100. In each case, the incubation lasted 1 h at room temperature. Between incubations, vibratome sections were first washed for 2 h or longer in the same solution as that used in diluting the antisera for the previous incubation and then treated for 30 min with 5% normal sheep serum in the same solution as that used in diluting the antisera for the following incubation. *d*, HRP-filled terminals in partially denervated sympathetic ganglia. The sympathetic chain was severed above the seventh ganglion 5 days before preganglionic fibres were filled orthodromically with HRP. This denervation procedure removes cholinergic terminals around B cells, but leaves intact cholinergic terminals on C cells and peptidergic terminals for the late slow e.p.s.p. Note that very few terminals are found near B cells. To fill preganglionic fibres, a fresh cut was made in the chain below the seventh ganglion and the cut end was taken up in a suction electrode containing 30% HRP and 3% lyssolecithin in dH_2O . Sixteen h later, the ganglia were fixed with 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 1 h, cut into 50- μm sections on a vibratome, and reacted in a mixture of 0.5 mg ml^{-1} DAB and 0.0009% H_2O_2 in 0.1 M phosphate-buffered saline for 10–15 min. B and C in *a*, *d* indicate B cells and C cells respectively, as judged from their sizes. In all cases, the sections were unstained, apart from the end-product of the peroxidase reactions.

Table 1 Distribution of LHRH-positive boutons in sympathetic ganglia

	Small cells ($<28\ \mu\text{m}$ in diameter)			Medium cells ($>28\ \mu\text{m}$, $<42\ \mu\text{m}$ in diameter)			Large cells ($>42\ \mu\text{m}$ in diameter)		
	No. stained	Total no.	% Stained	No. stained	Total no.	% Stained	No. stained	Total no.	% Stained
Normal	640	792	81	307	653	47	14	203	7
Unilaterally denervated	84	451	19	164	557	29	177	356	50
Bilaterally denervated	13	502	2.6	85	508	17	55	203	27

50- μm thick sections of ganglia stained immunohistochemically with PAP and Sternberger's antisera for mammalian LHRH (see Fig. 1 legend) were examined with a Zeiss microscope and a $\times 40$ water immersion lens. Diameters of cells surrounded with LHRH-positive boutons were measured, as were diameters of cells which clearly had no staining in their vicinity. (Using such criteria, the latter cell type is probably somewhat under-represented.) 'Diameters' for oblong cells were estimated as the geometric mean of the length and width of each cell. No corrections were made on account of the thickness of the sections. 'No. stained', number of cells surrounded by LHRH-positive terminals; 'Total no.', the sum of the number of cells surrounded by LHRH-positive terminals and the number of cells clearly void of stained terminals in their vicinity; '% Stained', the ratio of no. stained to total no. 'Normal' includes sections of ninth and tenth ganglia from five frogs; 'Unilaterally denervated' includes sections of ninth and tenth ganglia with their ipsilateral input from the seventh and eighth spinal nerves removed for 10 days (three frogs); and 'Bilaterally denervated' includes sections of ninth and tenth ganglia from two frogs whose seventh and eighth rami on both sides had been cut for 10 days.

The principal neurones in the ninth and tenth ganglia can be divided into two groups⁵: B cells and C cells. B cells are larger (with diameters from ~ 30 to $\sim 70\ \mu\text{m}$). They send out axons that conduct at velocities of about $2\ \text{m s}^{-1}$. C neurones are smaller (with diameters from ~ 20 to $\sim 45\ \mu\text{m}$) and their axons conduct at speeds $\sim 0.2\ \text{m s}^{-1}$. Both B and C cells give late slow e.p.s.ps on stimulation of the seventh and eighth spinal nerves, which carry most of this non-cholinergic input. Cholinergic inputs to these cells, however, are segregated anatomically⁶. B cells are innervated by cholinergic fibres from the chain above the seventh ganglion whereas C cells receive cholinergic input through the seventh and eighth spinal nerves (Fig. 2).

Although physiological studies have shown that most B and C cells give late slow e.p.s.ps on stimulation of the seventh and eighth spinal nerves^{3,6}, immunohistochemical experiments reveal that LHRH-positive synaptic boutons are mainly around C cells (14 frogs). Cell counts on 50- μm thick sections of ganglia from five frogs showed that 640 out of 792 small cells (with diameters $<28\ \mu\text{m}$) were surrounded by LHRH-positive synaptic boutons, whereas only 14 out of 203 large cells (with diameters $>42\ \mu\text{m}$) were surrounded by LHRH-positive terminals (Table 1). As physiological studies have shown that almost all cells larger than $45\ \mu\text{m}$ in diameter are B cells, whereas cells smaller than $30\ \mu\text{m}$ in diameter are most likely C cells, it seems that most, if not all, LHRH-positive nerve terminals are apposed to C cells.

This preferential staining of synaptic boutons around C cells was confirmed with two different approaches. First, we eliminated the cholinergic input to B cells by cutting the sympathetic chain above the seventh ganglion, identified B cells which gave the late slow e.p.s.p. but no cholinergic synaptic potentials, and serial-sectioned through those cells to look for remaining terminals using an electron microscope. Of the three B cells studied, none had terminals directly apposed to its membrane. Second, we again cut the chain above the seventh ganglion to remove the cholinergic innervation to B cells, leaving intact the cholinergic innervation to C cells plus peptidergic inputs to both B and C cells (Fig. 2). Then we used horseradish peroxidase (HRP) to fill terminals of preganglionic fibres arising from the seventh and eighth spinal nerves. Again, HRP-filled terminals were found around C cells, but hardly any filled terminals were found on B cells (Fig. 1d). Together, these anatomical approaches suggest that synaptic boutons containing LHRH-like immunoreactivity are almost exclusively around C cells. The similarity between the pattern of distribution of LHRH-positive boutons and that of HRP-filled terminals around C cells raises the question of whether acetylcholine and LHRH might exist in the same synaptic boutons, as has been suggested in some systems⁷.

To test further whether these LHRH-positive boutons arise from preganglionic fibres responsible for the late slow e.p.s.p., we removed most of those fibres from the ganglia by cutting the

seventh and eighth rami (Fig. 2). Previous physiological and anatomical studies have shown that after the preganglionic fibres are cut, synaptic transmission fails and nerve terminals of cut axons degenerate in a few days, but the ganglion cells retain their membrane potentials and give impulses. As expected, immunohistochemical staining of denervated ganglia showed that the staining for LHRH-like immunoreactivity was

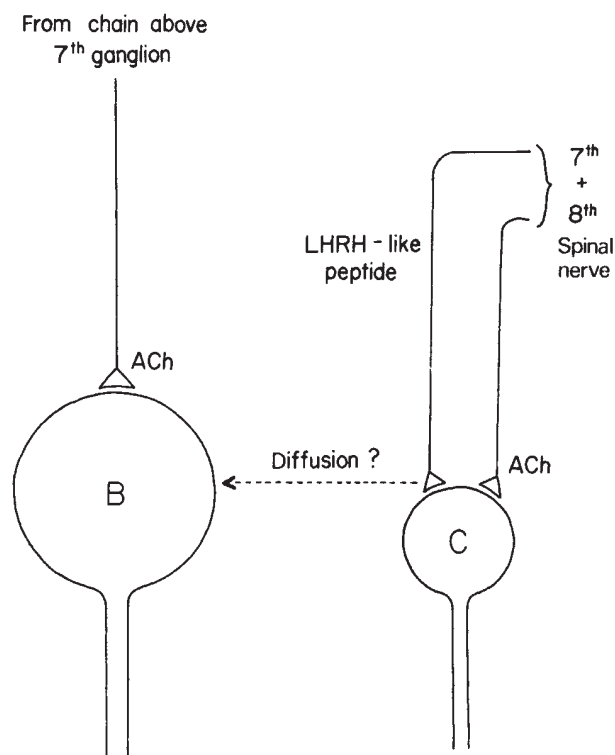


Fig. 2 Scheme of innervation of principal cells in ninth or tenth ganglia, the last two ganglia in the sympathetic chain. B cells are larger (~ 30 to $\sim 70\ \mu\text{m}$ in diameter), whereas C cells are generally smaller (with diameters from ~ 20 to $\sim 45\ \mu\text{m}$ possibly down to 10 – $15\ \mu\text{m}$). Cholinergic axons for B neurones reach these ganglia through the sympathetic chain above the seventh ganglion, whereas preganglionic fibres for C neurones come through the seventh and eighth spinal nerves. The majority of LHRH-positive preganglionic axons for all neurones in the ninth and tenth ganglia also enter through the seventh and eighth nerves. Peptidergic input to B neurones is represented by dotted lines, because very few LHRH-positive boutons are found near large B neurones. It is not yet known whether acetylcholine and the LHRH-like peptide exist in the same nerve terminals around C cells.

essentially normal in the ninth and tenth ganglia after the chain was severed above the seventh ganglion (three frogs). On the other hand, cutting the seventh and eighth rami greatly reduced the staining (Fig. 1c). Rather unexpectedly, the distribution of staining in these denervated ganglia turned out to be drastically different from the normal pattern. Most of the cells surrounded by LHRH-positive boutons are large B cells (Table 1). The residual staining around C cells may be accounted for by a small percentage of peptidergic input from the contralateral seventh and eighth spinal nerves, as from previous electrophysiological experiments we knew that such contralateral contributions normally exist and that they increase after the ipsilateral input is removed, possibly as a result of sprouting⁸. Indeed, in two frogs whose seventh and eighth rami were cut on both sides, only 2.6% of small cells were surrounded by LHRH-positive boutons (Table 1), compared with 19% in unilaterally denervated frogs or 81% in the unoperated frog. Therefore, cutting preganglionic fibres from the seventh and eighth rami almost completely removed staining around C cells. The staining around large cells after denervation could be due to sprouting of the remaining few LHRH-positive fibres, which occasionally arise from the ninth spinal nerve, uptake of LHRH-positive materials into cholinergic nerve terminals, or other factors. Further studies are necessary to answer this question.

LHRH-like immunoreactivity has been found in synaptic boutons, as seen by light microscopy, in sympathetic ganglia. Further, denervation experiments suggest that these LHRH-positive synaptic boutons are probably the preganglionic nerve terminals for the late slow e.p.s.p. This considerably strengthens our hypothesis that the transmitter for the late slow e.p.s.p. in frog sympathetic ganglia is a LHRH-like peptide. Although the late slow e.p.s.p. is present in both B and C cells, LHRH-positive synaptic boutons are mainly around C cells. Therefore, whereas C cells may receive peptidergic transmitters from synaptic boutons in the vicinity of their cell bodies, the late slow e.p.s.p.s. recorded in B cells must be largely due to diffusion of the LHRH-like peptides from terminals around C cells. The delayed and variable onset of the late slow e.p.s.p. recorded in different B cells is compatible with the suggestion that B cells receive peptidergic transmitters that are released at a distance. This result implies that inactivation of this peptidergic transmitter must be relatively slow. Indeed, if an antagonist of LHRH is ejected by pressure on to a B cell anytime during a late slow e.p.s.p., the slow synaptic potential is truncated right away⁸, suggesting that the slowness of this response is partly due to the prolonged presence of transmitters after they are released on nerve stimulation. The fact that sympathetic ganglia contained two anatomically distinct types of peptidergic innervation, together with their relative structural simplicity and easy access, makes them a promising model for detailed analysis of peptidergic transmission.

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Do human platelets have opiate receptors?

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In their study of prostaglandin E_1 (PGE_1)-sensitive adenylate cyclase (AC) in rat brain homogenates, Collier and Roy¹ claimed that the activity of this enzyme is inhibited by opiates. They also proposed that opiates exert their analgesic and allied effects by inhibiting AC of neurones that are normally stimulated by E prostaglandins². Studies using neuroblastoma × glioma hybrid cells^{3,4} supported this hypothesis. However, subsequent studies with the mammalian brain^{5,6} and rat brain tissue slices^{7,8} yielded conflicting results. PGE_1 also inhibits platelet aggregation⁹, probably through activation of platelet AC¹⁰. Gryglewski *et al.*¹¹ showed that morphine inhibits the anti-aggregating effect of PGE_1 on ADP- and adrenaline-induced platelet aggregation, and suggested that the inhibition by morphine is mediated through platelet AC activity. We report here our attempts to reproduce the results of Gryglewski *et al.* and our examination of the effect of morphine on PGE_1 -sensitive AC activity in platelet lysates and on PGE_1 -induced accumulation of cyclic AMP in intact platelets. The possible existence of opiate receptors in platelets was also assessed by direct binding studies with 3H -etorphine. In contrast to Gryglewski *et al.*¹¹, we could not detect any effect of opiates on the aggregation of human platelets, nor did we find any other evidence supporting the presence of opiate receptors in these cells. Thus we conclude that the presence of opiate receptors in human platelets is unlikely.

Blood collection and preparation of citrated platelet-rich and platelet-poor plasmas were done according to Han and Ardlie¹². Platelet aggregation was measured by the turbidometric technique of Born¹³, as described previously¹⁴. Human platelet lysates were prepared by a modification of the method of Jakobs *et al.*¹⁵, as described previously¹⁴. Homogenates of rat corpus striatum (10% w/v in 0.32 M sucrose 10 mM Tris-HCl pH 7.6) and P_2 -pellets were prepared according to Gray and Whittaker¹⁶.

Adenylate cyclase activity in platelet lysates was determined as described previously by measuring the conversion of [α - ^{32}P]ATP (Amersham) to ^{32}P -cyclic AMP by the method of Salomon¹⁷. Cyclic AMP accumulation in intact 3H -adenine (Negev)-prelabelled platelets was as described previously¹⁷.

Table 1 The effect of PGE_1 and morphine on PGE_1 -sensitive AC activity in platelet lysates

Additions	None	Morphine (13.3 μ M)	PGE_1 (0.35 μ M)	PGE_1 + morphine (0.35 μ M + 13.3 μ M)
			cAMP (pmol per mg protein per 10 min)	
Donor 1	86	110	2,103	2,108
Donor 2	116	110	2,122	2,046
Donor 3	106	87	684	643
Donor 4	65	71	1,395	1,152
Mean $\pm$ s.d.	93.2 $\pm$ 13.0	94.5 $\pm$ 11	1,576 $\pm$ 395	1,487 $\pm$ 411
P value		NS		NS

Each value is the average of duplicate determinations. NS, not significant by paired *t*-test.

Binding of ^3H -etorphine (Amersham) to platelet lysates was measured as described for rat brain homogenates¹⁸. The amount of lysate protein was 0.05–0.45 mg per assay. ^3H -etorphine concentration was 3.0 nM and incubations were performed in a final volume of 250 μl for 30 min at 37 °C. The experiments were repeated 3–5 times with platelet preparations derived from several healthy volunteers who had not taken any drugs for at least 1 week before blood collection.

PGE_1 , 0.02–0.34 μM , inhibited ADP-induced aggregation in a dose-dependent manner. In five experiments we used 0.14 μM PGE_1 (the concentration used by Gryglewski *et al.*¹¹). At this concentration PGE_1 inhibited ADP-induced platelet aggregation by $70.5\% \pm 1 \text{ s.e.m.}$ In these conditions we tested a wide range of morphine concentrations (13.2–53 μM). No significant effect on the anti-aggregating properties of PGE_1 could be demonstrated.

Stimulation of platelet AC by PGE_1 in the concentration range of 0.01–0.35 μM was dose dependent. At the highest concentration (0.35 μM), PGE_1 stimulated AC activity by about 6–20-fold (Table 1). We tested platelet preparations from four donors but could not demonstrate any inhibitory effect of morphine (13.3 μM) on basal (unstimulated) or PGE_1 -sensitive AC activity (Table 1). The concentration of morphine selected for this experiment was that reported by Gryglewski *et al.*¹¹ to be optimal in their experiments.

Table 2 The effect of PGE_1 and morphine on the intracellular accumulation of ^3H -cyclic AMP in intact platelets prelabelled with [$2\text{-}^3\text{H}$]adenine

Additions	Morphine (μM)		
	0	13.3	26
	^3H -cyclic AMP (c.p.m.)		
None	85 ± 2	93 ± 5	98 ± 2
PGE_1 (0.14 μM)	496 ± 25	525 ± 19	540 ± 28

Results are given in c.p.m., mean $\pm$ s.d. ($n = 4$).

To eliminate the possibility that the inhibitory effect of morphine may not be demonstrable at saturating PGE_1 concentrations, we also tested its effect at submaximal concentrations of PGE_1 . At 0.04 μM PGE_1 , AC activity was stimulated over 65–490 pmol cyclic AMP per mg protein per 10 min. Addition of a wide range of morphine concentrations (1.33–79.8 μM) did not inhibit the basal or the PGE_1 -stimulated enzyme activity. The same results were obtained with lysates preincubated with morphine (2 min, 30 °C) before the AC assay.

Similar negative observations were also made with intact platelets. PGE_1 (0.14–0.7 μM) stimulated cyclic AMP accumulation by 1.8- to 10.1-fold over the unstimulated controls. The addition of morphine (2.6–26 μM) did not affect the unstimulated controls. The addition of morphine (2.6–26 μM) did not affect the stimulatory action of PGE_1 . A typical experiment in which the lowest concentration of PGE_1 (0.14 μM) was used in combination with 13.3 or 26 μM morphine is given in Table 2.

In direct binding studies of ^3H -etorphine to platelet lysate preparations, we failed to demonstrate any specific binding. The difference between the amount of ^3H -etorphine bound in the absence or presence of 10 μM levorphanol was not significantly different from zero (four duplicate determinations performed on each platelet preparation obtained from four donors). Simultaneously with these determinations, and in identical experimental conditions, we also measured the binding of ^3H -etorphine to a particulate fraction derived from rat corpus striatum as a control tissue. The content of ^3H -etorphine binding sites in this brain preparation was $315 \pm 45 \text{ fmol per mg protein}$ ($n = 4$). Binding of ^3H -etorphine in the presence of 10 μM levorphanol did not exceed 25–40% of the amount bound in the absence of the unlabelled competing ligand.

In conclusion, the inverse relationship of cyclic AMP levels and aggregability of human platelets is now well documented¹⁹, as PGE_1 , which stimulates platelet AC¹⁰, also antagonizes ADP-

induced aggregation⁹. Furthermore, this relationship also seems to hold for nonspecific agents such as heparin and dextran sulphate, recently demonstrated by us to inhibit PGE_1 -stimulated AC on the one hand and to augment platelet aggregation on the other^{14,20–22}. Although opiates act as inhibiting agonists of AC in some cell types of neural origin, we are so far unable to support the suggestion made by Gryglewski *et al.*¹¹ that 'AC is the common target for PGE_1 and morphine in platelets as it is in neurones'. We therefore suggest that the presence of opiate receptors in human platelets remains questionable.

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Induction of diabetes by cumulative environmental insults from viruses and chemicals

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Encephalomyocarditis virus (EMC) induces diabetes in certain inbred strains of mice by infecting and destroying pancreatic β cells^{1,2}, the severity of the diabetes depending on the number of β cells destroyed³. In strains of mice resistant to EMC-induced diabetes, insufficient β cells are damaged to alter glucose homeostasis^{3,4}. However, diabetes can be produced in many species by streptozotocin, a highly specific β -cell toxin⁵. Here, we used concentrations of streptozotocin that did not produce diabetes, but reduced the β -cell reserve. When strains of mice normally resistant to EMC-induced diabetes were first treated with sub-diabetogenic doses of streptozotocin, then infected with EMC virus, diabetes developed. Furthermore, when mice were infected with viruses such as Coxsackie B3 and B5, which ordinarily produce little if any β -cell damage⁶, diabetes developed if the mice were first treated with sub-diabetogenic doses of streptozotocin. These findings suggest that diabetes may result from cumulative β -cell damage induced by sequential environmental insults.

Male mice, 4–6 weeks old, weighing 20–25 g (Jackson Laboratories or NIH), were used in these experiments. A highly diabetogenic variant of EMC virus, designated D, and a non-diabetogenic variant, designated B, were prepared in secondary mouse embryo cells as described elsewhere⁷. Unless otherwise indicated, the D variant was used in all experiments. Coxsackieviruses B3 (Nancy strain) and B5 (Faulkner strain) (both from American Type Culture Collection) were passaged in cultures enriched with murine pancreatic β cells⁸. The preparation of fluorescein-labelled anti-EMC antibody and the detection of infected islet cells were carried out as described elsewhere^{3,4}.

Mice were injected intraperitoneally with a sub-diabetogenic dose of streptozotocin (1 mg per mouse, mixed anomers, Sigma lot 29C-0009), which did not significantly alter glucose homeostasis in preliminary experiments. Twelve days after administration of streptozotocin, mice were infected intraperitoneally with virus. Each animal was then bled four times; glucose from non-fasting animals was measured 7 and 14 days after infection and single-point 60-min glucose tolerance tests were performed 10 and 17 days after infection. Uninfected animals and animals given only streptozotocin were bled at the same time. The weighted results were expressed as the glucose index⁹. An animal was considered diabetic if its glucose index was equal to or exceeded 3 s.d. above the mean of 124 uninfected mice (mean $\pm$ s.d. = 163 ± 23).

Table 1 shows that the glucose index and percentage of animals developing diabetes was increased if diabetes-prone strains of mice^{4,10} were treated with sub-diabetogenic doses of streptozotocin before infection with EMC virus. The most striking results were obtained with NIH/Swiss mice in which the glucose index increased from 223 in mice receiving only EMC virus to 442 in mice receiving both streptozotocin and EMC virus, and the percentage of animals developing diabetes rose

from 28 to 92. Increases in the glucose index were also observed in SJL/J and NFS/N mice. The enhancement in diabetes-prone mice suggested that strains of mice which were ordinarily resistant to EMC-induced diabetes^{4,10} might become diabetic if first treated with sub-diabetogenic doses of streptozotocin. As seen in Table 1, 18%, 30%, 53% and 100% of C3H/HeJ, C57BL/6J, CBA/J and AKR/J mice, respectively, developed diabetes when treated with streptozotocin and infected 12 days later with EMC virus.

Treatment of mice with a low dose of streptozotocin also led to the induction of diabetes by viruses which ordinarily did not elevate blood glucose levels. Table 2 shows that Coxsackieviruses B3 and B5 produced diabetes in animals which had been treated with a sub-diabetogenic dose of streptozotocin, whereas in untreated animals these viruses produced little if any diabetes. However, the non-diabetogenic B variant of EMC virus⁷, did not produce diabetes in either streptozotocin-treated or untreated mice.

To determine whether streptozotocin enhanced virus-induced diabetes by increasing the susceptibility of islet cells (for example, by immunosuppressing the host)¹¹, the number of EMC-infected islet cells was determined by staining sections of pancreas with fluorescein-labelled anti-EMC virus antibody^{3,4}. Table 3 shows that approximately the same or slightly fewer islet cells were infected in streptozotocin-treated mice as in untreated mice. This suggests that streptozotocin is acting by decreasing the β -cell reserve rather than by increasing the susceptibility of β cells to infection. In fact, microscopic examination of sections of pancreas from mice treated with streptozotocin (1 mg per mouse) showed that some β cells had been destroyed. Furthermore, quantification by radioimmunoassay revealed that the insulin content of the pancreas of mice treated with 1 and 2 mg of streptozotocin was reduced by 15% and 48%, respectively, 12 days later.

Table 1 Enhancement of virus-induced diabetes by treatment with sub-diabetogenic doses of streptozotocin

Mouse strain	Streptozotocin		EMC virus		Streptozotocin and EMC virus	
	Glucose index*	Diabetic (%)†	Glucose index	Diabetic (%)	Glucose index	Diabetic (%)
Diabetes-prone						
NIH/Swiss	142 $\pm$ 29	0	223 $\pm$ 89	28	442 $\pm$ 153	92
SJL/J	166 $\pm$ 16	0	333 $\pm$ 113	76	466 $\pm$ 126	89
NFS/N	172 $\pm$ 15	0	358 $\pm$ 47	100	448 $\pm$ 80	100
Diabetes-resistant						
C3H/HeJ	186 $\pm$ 18	0	151 $\pm$ 23	0	202 $\pm$ 30	18
C57BL/6J	126 $\pm$ 24	0	152 $\pm$ 33	6	203 $\pm$ 89	30
CBA/J	185 $\pm$ 17	0	183 $\pm$ 24	0	234 $\pm$ 38	53
AKR/J	189 $\pm$ 39	13	172 $\pm$ 29	0	345 $\pm$ 95	100

Each mouse was given 1 mg of streptozotocin and 12 days later infected with 1.0×10^4 plaque-forming units (PFU) of the D variant of EMC virus.

* Approximately 20 mice were tested in each group (mean $\pm$ s.d.).

† Percentage of mice with a glucose index 3 s.d. above the mean of uninfected controls.

Table 2 Induction of diabetes by Coxsackieviruses in mice treated with sub-diabetogenic doses of streptozotocin

Mouse strain	Virus	Streptozotocin		Virus		Streptozotocin and virus	
		Glucose index*	Diabetic (%)†	Glucose index	Diabetic (%)	Glucose index	Diabetic (%)
SJL/J	Cox B3	149 $\pm$ 35	0	198 $\pm$ 28	18	241 $\pm$ 43	59
NFS/N	Cox B3	180 $\pm$ 22	0	158 $\pm$ 18	0	285 $\pm$ 76	76
NFS/N	Cox B5	180 $\pm$ 22	0	166 $\pm$ 13	0	267 $\pm$ 58	67
SJL/J	EMC(B)	177 $\pm$ 12	0	175 $\pm$ 14	0	191 $\pm$ 17	0

Each mouse was given 1 mg of streptozotocin and 12 days later infected with 1.0×10^5 PFU of Coxsackievirus B3 or B5, or 1.0×10^6 PFU of the B variant of EMC virus.

* Approximately 20 mice were tested in each group (mean $\pm$ s.d.).

† Percentage of mice with a glucose index 3 s.d. above the mean of uninfected controls.

In the normal animal there are enough β cells to maintain glucose homeostasis—only when the β -cell reserve is sufficiently depleted does hyperglycaemia develop⁵. Our experiments show that, by reducing the β -cell reserve with a sub-diabetogenic dose of streptozotocin, it is possible to produce diabetes in strains of mice which are usually resistant to EMC-induced diabetes. In previous experiments with EMC virus, we found that over 40% of the β cells in the pancreas of diabetes-prone SJL/J mice contained viral antigens, as opposed to less than 12% of β cells from diabetes-resistant CBA/J, AKR/J and C57BL/6J mice⁴.

Table 3 Percentage of cells in islets of Langerhans containing viral antigens

Mouse strain	Pretreatment with streptozotocin*	(% infected islet cells) (days after infection)†		
		(2)	(3)	(4)
C57BL/6J	+	2	12	7
C57BL/6J	—	2	12	9
SJL/J	+	30	36	10
SJL/J	—	41	44	12

* Each mouse received 1 mg of streptozotocin intraperitoneally 12 days before infection with 1.0×10^4 PFU of the D variant of EMC virus.

† At 2, 3 and 4 days after infection, sections of pancreas from groups of three mice were stained with fluorescein-labelled anti-EMC antibody and the approximate number of cells containing viral antigens determined. At each time point, an average of 40 islets and over 2,000 cells were counted.

It was concluded that the latter strains did not develop diabetes because β -cell damage was insufficiently extensive. The experiments reported here show that neither a low dose of streptozotocin alone nor EMC virus alone produced diabetes, but the combined insult resulted in diabetes. Similarly, Cox-sackieviruses B3 and B5 produced relatively little β -cell damage in untreated animals, but when the β -cell reserve was reduced by a low dose of streptozotocin, the animals developed diabetes.

Depletion of β cells in mice by sub-diabetogenic doses of streptozotocin may provide a useful model for identifying other diabetogenic viruses, especially those which infect and destroy only minimal numbers of β cells. The enhancement of virus-induced diabetes by low doses of streptozotocin also raises the possibility that in humans a series of viral infections or other environmental insults (for example, chemicals, drugs, toxins), each producing some β -cell damage, finally results in overt insulin-dependent diabetes once the β -cell reserve has been sufficiently depleted.

Genetic factors are also known to play an important role in the development of insulin-dependent diabetes², but precisely how is not clear. If the result is a relative deficiency of β cells or an impaired capacity to regenerate damaged β cells, then viruses, chemicals or drugs might more readily produce diabetes in these already deficient individuals.

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Tolerance as an active process

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The clonal deletion model proposed by Burnet¹ and Lederberg² and expanded by Nossal³ was one of the first theories concerning the nature of tolerance to self constituents. The model proposed that during the maturation of lymphocytes into immunocompetent cells, there is a sensitive differentiation stage whereby contact with antigen results in specific inactivation of the cell. Experimental evidence indicates that neonatal or immature B lymphocytes are indeed different from adult lymphocytes in their extreme sensitivity to tolerance induction even at low antigen concentrations and with antigens that are normally immunogenic^{3–5}. The present study examines the mechanism of this tolerance phenomenon by determining whether or not tolerance of immature B cells is an active process and what specific interactions can induce this event. We used various putative inhibitors of tolerance induction in the splenic focus assay⁴ which examines the tolerance susceptibility of individual B cells. The results suggest that tolerance requires protein synthesis and that this process is initiated only after a minimum threshold affinity of binding occurs between antigen and cell-surface receptor with subsequent receptor interlinkage.

Nonimmune neonatal or adult spleen cells were injected intravenously into irradiated (1,300R) BALB/c recipients that had been primed with 0.1 mg of *Limulus polyphemus* haemocyanin (Hy) 6–12 weeks previously. At 16–18 h after transfer, fragment cultures derived from the recipient spleens were prepared and incubated for 18 h in Dulbecco's modified Eagle's medium (DMEM) with or without the presence of the tolerogen dinitrophenol-ovalbumin (DNP₁₀-OVA) at 10^{-6} M DNP⁴. To study the inhibition of tolerance induction, concentrations of putative inhibitors were chosen which, if removed after the first 18 h of culture and before antigenic stimulation, had no inhibitory effect on stimulation. Thus, by adding DNP₁₀-OVA in the presence or absence of the inhibitors for the first 18 h of culture, the effect of each inhibitor on tolerance could be determined. The DNP₁₀-OVA and/or inhibitors were washed out after 18 h and DMEM containing the antigen DNP₁₀-Hy (10^{-6} M DNP)⁴ was added. Culture supernatants were collected on days 10, 13 and 17 and analysed for the presence of anti-DNP antibody by a

Table 1 A requirement for protein synthesis during tolerance induction

Age of donor cells	Experimental group	% Of control response*
1–3 day neonatal	Tolerogen (DNP ₁₀ -OVA)	28.5 ± 7.0
1–3 day neonatal	Puromycin alone	87.5 ± 12.7
1–3 day neonatal	Tolerogen + puromycin	92.1 ± 3.8
10–12 week adult	Tolerogen (DNP ₁₀ -OVA)	100
10–12 week adult	Puromycin alone	77.0
10–12 week adult	Tolerogen + puromycin	77.0

Adult or neonatal spleen cells were transferred to carrier-primed recipients ($4\text{--}6 \times 10^6$ donor cells per recipient). Fragment cultures were incubated for 18 h in the presence or absence of the tolerogen DNP₁₀-OVA and in the presence or absence of the inhibitor puromycin ($25 \mu\text{g ml}^{-1}$ for adult groups, $20 \mu\text{g ml}^{-1}$ for neonatal groups). The tolerogen and/or inhibitor was/were washed out after 18 h and stimulated for 3 days with DNP-Hy (10^{-6} M DNP). Supernatants were collected on days 10, 13 and 17 and the frequency of anti-DNP-secreting clones was detected by RIA. Data are expressed as per cent of control response.

* Neonatal groups represent the means of three experiments ± s.e.m. Adult groups represent the average of two experiments.

solid-phase radioimmunoassay (RIA). The anti-DNP antibody was detected in RIA through the use of rabbit anti-mouse immunoglobulin followed by ^{125}I -labelled purified goat anti-rabbit immunoglobulin¹⁰.

To determine if tolerance of neonatal B cells represents an active process which would require a trigger, the effect of the protein biosynthesis inhibitor, puromycin, was studied. It was assumed that any effect on tolerance observed would be due to the effect of puromycin on the neonatal donor B cells, although there is a remote possibility that the highly irradiated cells also present in the fragment are involved in tolerance and, therefore, would be affected by puromycin. Table 1 shows that puromycin substantially inhibits the induction of tolerance by DNP₁₀-OVA resulting in stimulation and production of anti-DNP-secreting clones at a frequency close to that observed in control cultures. On the other hand, when adult B cells were assayed using the same experimental procedure, they were found to be resistant to tolerance induction as reported elsewhere^{4,5} and the addition of puromycin was, therefore, inconsequential. The requirement for protein biosynthesis during tolerance induction of neonatal B cells provides compelling evidence that tolerance is an active process and cannot be explained simply by passive phenomena such as receptor blockade^{11,12}.

Our next concern was the mechanism by which immature B cells are induced into the tolerance mode, including the antigen-binding prerequisites of the tolerance trigger. Although previous studies had indicated that multivalent antigens maximize tolerance induction^{4,13}, a requirement for multivalent interactions during tolerance had not been tested directly. Thus, the effect of various concentrations of DNP-L-lysine on DNP₁₀-OVA-induced tolerance was ascertained. Table 2 indicates that a 10-fold higher molar concentration of DNP-L-lysine as compared with the molar concentration of DNP present on DNP₁₀-OVA markedly inhibits tolerance induction. Inhibitory effects of equal molar concentrations and 10-fold lower concentrations of DNP-L-lysine can also be seen. This finding demonstrates the necessity for multivalent interactions between tolerogen and cell-surface receptors. As DNP-L-lysine can bind receptors with a high enough affinity to inhibit tolerance induced by DNP presented multivalently, it is inferred that binding alone is not sufficient to trigger tolerance but that receptor interlinkage must be achieved.

It is well known that the antibody products from most DNP-specific B cells can bind to the hapten trinitrophenol (TNP)^{14,15}. However, TNP antigens seem incapable of stimulating primary DNP-specific B cells^{14,15} or of tolerizing DNP-specific immature B cells⁴. Therefore, it was of interest to determine if various concentrations of TNP-L-lysine could inhibit tolerance induction by DNP₁₀-OVA (Table 2). Similar to the experiments using DNP-L-lysine, a 10-fold higher molar concentration of TNP-L-lysine as compared with the molar concentration of DNP on DNP₁₀-OVA resulted in almost complete inhibition of tolerance. However, equal and 10-fold lower concentrations of TNP-L-lysine resulted in little or no

inhibition of tolerance induction, indicating that a higher ratio of TNP to DNP is necessary to inhibit tolerance than when the homologous hapten is used as an inhibitor. This suggests that TNP-L-lysine binds to DNP-specific B cells but at a lower affinity than DNP-L-lysine. As TNP-L-lysine does bind to the receptors of DNP-specific B cells, the failure of TNP antigens to trigger DNP-specific B cells is probably directly related to the affinity with which it is bound by the cell's receptors.

Table 3 Inhibition of DNP₁₂-OVA-induced tolerance with TNP₁₇-BSA

Experimental treatment	% Of control response*
DNP response	
Tolerogen alone (DNP ₁₀ -OVA, 10^{-6} M)	27.8 ± 7.8
Tolerogen (DNP ₁₀ -OVA, 10^{-6} M) + TNP ₁₇ -BSA (10^{-5} M)	89.6 ± 10.5
TNP ₁₇ -BSA (10^{-5} M)	100
TNP response	
Tolerogen (TNP ₁₇ -BSA, 10^{-6} M)	33.3

For the DNP response, the same procedure was used as described in Table 1 legend except TNP₁₇-BSA was used as inhibitor. For the TNP response, TNP₁₇-BSA was used as tolerogen and TNP₁₀-Hy (10^{-6} M) as antigen.

* Results represent the mean ± s.e.m. of three experiments. The results represent the average of two experiments where the s.e.m. is omitted.

To test directly the postulate that TNP antigens bind to DNP-specific B cells but fail to trigger these cells because the affinity of such binding is too low, an excess of TNP presented multivalently with bovine serum albumin (TNP₁₇-BSA) was tested for its ability to inhibit DNP₁₀-OVA-induced tolerance. As shown in Table 3, TNP₁₇-BSA inhibits DNP₁₀-OVA-induced tolerance demonstrating its ability to bind to DNP-specific B cells yet its inability to tolerize these cells. Also included in Table 3 are data which show that the same preparation of TNP₁₇-BSA is an effective tolerogen for TNP-specific cells. The ability to distinguish between two closely related haptens demonstrates that the tolerance trigger is an extremely specific one and that this specificity apparently depends on a minimum threshold affinity of binding between tolerogen and receptor. Below this threshold the tolerogen may not bind to the receptor long enough to achieve stable interlinkage of receptors¹⁶.

If one compares the analyses of the apparent binding requisites of stimulation with the present findings on tolerance, obvious parallels can be drawn between the two processes. First, despite a few reports to the contrary¹⁷⁻¹⁹, monovalent haptens seem unable to induce stimulation^{13,20,21}, and in fact, can inhibit stimulation induced by the same hapten presented multivalently¹⁶. Thus, stimulation, like tolerance, apparently requires multivalent determinant presentation and receptor interlinkage^{13,16,20-22}. Whether or not this is analogous to the receptor patching-capping-endocytosis cycle induced by anti-immunoglobulin²³ or some antigens²³⁻²⁵ is unclear as considerably greater concentrations of either anti-immunoglobulin or antigen are required to induce this cycle than are required to induce either stimulation or tolerance²⁵. In any event, more than receptor modulation is required to achieve both stimulation and tolerance as protein synthesis is required (ref. 26 and this work), a requirement not found necessary for the patching-capping-endocytosis cycle²³.

Similarly, both the tolerance and the stimulation seem to be dependent on a minimum threshold affinity of binding between antigen and receptor. Earlier studies demonstrating the affinity dependence of stimulation showed that increasing concentrations of antigen above 5×10^{-7} M did not stimulate a higher frequency of cells nor did it stimulate more cells producing lower-affinity antibody^{15,16}. Clearly, antigen-binding cells did not correlate with cells capable of being triggered. Correspondingly, as mentioned previously, it was found that TNP on an

Table 2 Inhibition of the tolerance trigger by monovalent haptens

Experimental treatment	% Of control response*	
	Neonates	Adult
Tolerogen alone (DNP ₁₀ -OVA, 10^{-6} M DNP)	27.8 ± 9.3	100 ± 0
Tolerogen + 10^{-7} M DNP-L-lysine	60.4	77.0
Tolerogen + 10^{-6} M DNP-L-lysine	77.3 ± 9.1	100 ± 0
Tolerogen + 10^{-5} M DNP-L-lysine	95.8 ± 3.4	85.2 ± 8.7
Tolerogen + 10^{-7} M TNP-L-lysine	25.0	Not done
Tolerogen + 10^{-6} M TNP-L-lysine	53.5 ± 10.4	100
Tolerogen + 10^{-5} M TNP-L-lysine	94.3 ± 2.9	100

The same procedure was used as described in Table 1 legend except that the indicated concentrations of monovalent haptens were used as inhibitors.

* Results of three experiments ± s.e.m. Results represent the means of two experiments where s.e.m. is omitted.

appropriate carrier could not activate DNP-specific cells even though the antibody products cross-react^{13,14}. Therefore, the stimulation trigger, like the tolerance trigger, is an extremely specific one that seems to be a consequence of the requirement for a minimum affinity of binding between the antigen and receptor. It should be emphasized that stimulation is a more complex process than tolerance. Stimulation involves not only antigen-receptor interaction but also interactions with other cell-surface receptors and/or cell types (for example, macro-

phages, T cells). Thus, these experiments on stimulation are open to other interpretations. In comparison, the tolerance trigger seems to be relatively simple, involving only antigen-receptor interactions. Thus, for the first time it has been possible to affix a requirement for both multivalency and a minimum binding affinity to the triggering of B cells by antigen.

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Major anti-paternal alloantibody induced by murine pregnancy is non-complement-fixing IgG1

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Maternal humoral immune responses against antigens of the genetically alien embryo have been reported in several mammalian species, including man, although little is known of the biological relevance of this phenomenon. In the mouse, only females of certain inbred strains mated repeatedly with an allogeneic male produce antibody directed against paternally inherited fetal histocompatibility antigens, as assessed by haemagglutination techniques^{1–4}. It has been suggested that this characteristic of the female is associated with the H-2^b haplotype³, although some reports indicate that it also extends to other H-2 types⁵. Potentially deleterious complement-dependent cytotoxicity, albeit at low levels, has been claimed to be associated with this alloantibody, but we have been unable to detect any such activity in a large number of maternal sera⁶. Four IgG isotypes (IgG1, IgG2a, IgG2b and IgG3) have been identified and shown to occur in the serum of normal animals^{7,8}. Despite their similar physicochemical properties, which complicate purification procedures, the availability of immunoglobulin-secreting plasmacytomas has made possible the preparation of isotype-specific antisera^{9,10}. Using these antisera in a modified haemadsorption assay¹¹, we have now demonstrated that the major alloantibody response induced by murine pregnancy involves the non-complement-fixing IgG1 subclass. This is a noncytotoxic antibody with potentially protective (enhancing) properties¹².

Mature C57BL (H-2^b) inbred female mice were caged with fertile CBA/Ca (H-2^k) males and pregnancies recorded following observation of vaginal plugs. Between 1 and 7 days after birth of their third litter, females were bled by cardiac puncture and the serum stored at –20 °C. A group of C57BL animals were injected intraperitoneally (i.p.) with 3×10^7 spleen cells from CBA/Ca males in 0.2 ml of saline, at weekly intervals for 5 weeks. Fourteen days after the final injection, animals were bled by cardiac puncture and serum stored at –20 °C. The IgG subclass of the alloantibody response was determined by a modified haemadsorption assay¹¹, using sheep red blood cells (SRBCs) coated with rabbit anti-immunoglobulin, anti-IgG1 or

anti-IgG2 (Bionetics). Fibroblasts obtained by trypsinization of CBA/Ca 14-day embryos were cultured in immunofluorescence trays (Sterilin). After incubation with serial double dilutions of sera, plates were incubated with coated SRBCs. The presence of alloantibody was indicated by SRBC binding to the fibroblasts.

The sera from all nine multiparous females exhibited titres of alloantibody of the IgG1 subclass equivalent to the titres obtained with total immunoglobulin reagent (Fig. 1a) This indicates that approximately all the alloantibody present can be accounted for by IgG1 alloantibody. Although IgG2 alloantibody was detected in seven of the sera, this was present at only 1/32 to 1/8 of the levels of the IgG1 alloantibody. To compare this response with one induced by a well-documented route of immunization, sera from animals injected i.p. with allogeneic spleen cells were analysed. As shown in Fig. 1b, in three of the six sera the immunoglobulin levels could largely be accounted for by the IgG2 alloantibody (sera nos 4–6). In all cases, alloantibody of the IgG1 subclass was also detected, ranging from titres equal to those of the IgG2 alloantibody down to 1/4 of these levels. The finding in pregnancy of IgG1 alloantibody levels largely equivalent to those of the total immunoglobulins made it unnecessary, for the present purposes, to assess the precise level of any IgG3 response, especially as this is also believed to be a non-complement-fixing subclass. No cytotoxic activity was detected in any of the maternal sera.

The finding that the major alloantibody response directed against the paternally inherited fetal histocompatibility antigens in multiparous females is of the IgG1 subclass, and that no complement-dependent cytotoxicity is detectable in these sera, suggests that it represents a non-complement-fixing IgG1 subclass. All IgG1 antibodies have been assumed to be unable to fix complement^{10,13}, although a cytotoxic complement-fixing IgG1 antibody has recently been demonstrated in mice injected intravenously with SRBCs¹⁴. The alloantibody induced by i.p. immunization was cytotoxic, and this seems attributable to the predominance of IgG2 alloantibody. Although seven of the multiparous females also produced IgG2 alloantibody the levels were clearly too low to effect cytotoxicity. It is interesting that the levels of IgG1 antibody in the immunized mice were similar to those in the pregnant females, for this suggests there may be a selective suppression of complement-fixing IgG2 antibody in pregnancy.

We know of no comparable data on antibody subclass distribution in pregnancy for other species. In the human, it is well established that antibodies with cytotoxic activity (which are used as HLA-typing reagents) may be detected in up to 15% of primiparous and 60% of multiparous women. However, in almost all cases the titre of such cytotoxic antibody is very low

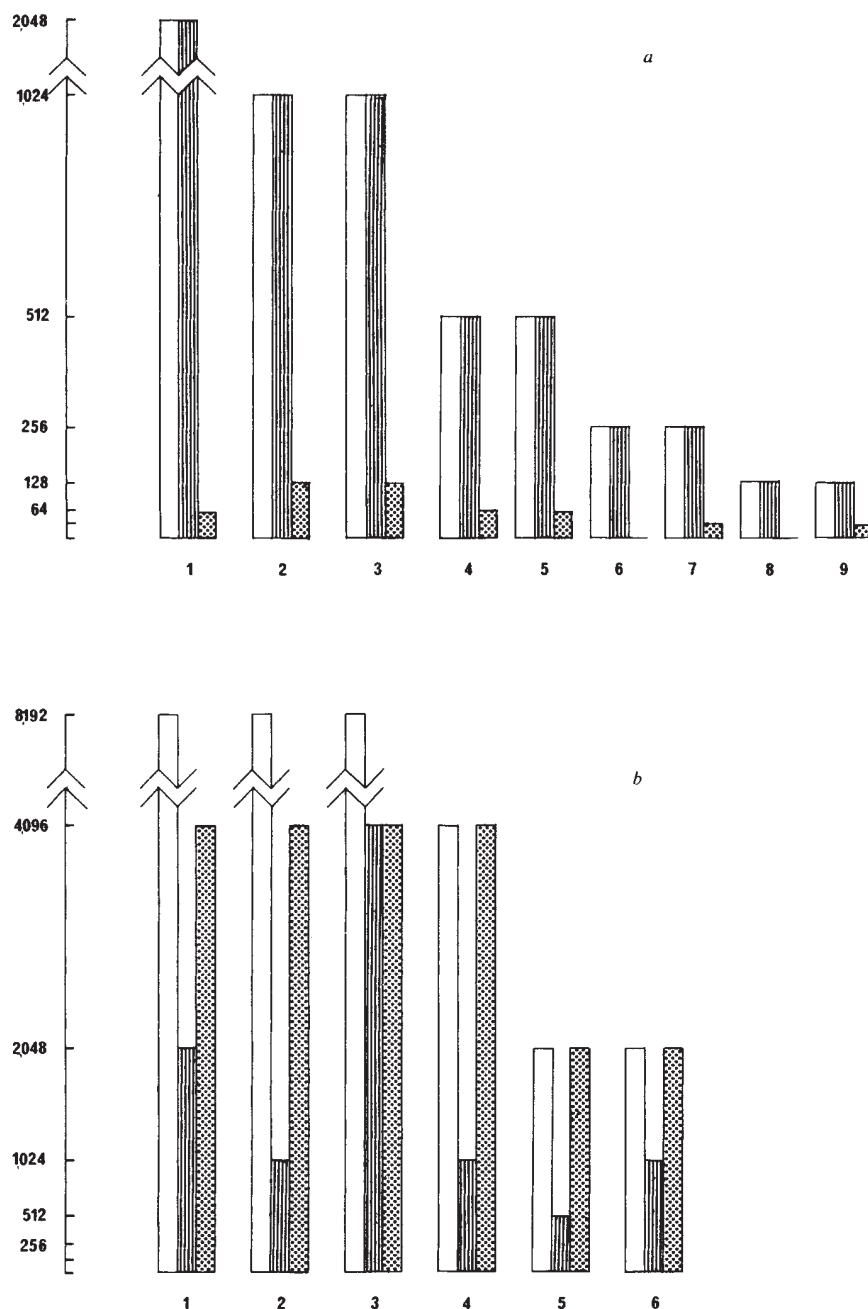


Fig. 1 Levels of anti-CBA/Ca (H-2^k) alloantibody as total immunoglobulin, IgG1 and IgG2 subclasses in sera from nine multiparous C57BL (H-2^b) females mated with CBA/Ca males (a) and six C57BL animals repeatedly immunized i.p. with CBA/Ca spleen cells (b). CBA/Ca fibroblasts (1×10^5 cells per well of immunofluorescence tray) were cultured for 24 h and, after removal of culture medium, serial double dilutions of test sera were incubated with the monolayer for 30 min. Sera were diluted with culture medium. Monolayers were then incubated for 30 min with SRBCs coated sequentially with mouse anti-SRBC sera and rabbit anti-mouse immunoglobulin, IgG1 or IgG2. After washing, monolayers were examined for SRBC binding. The final dilution of sera with which binding was obtained was considered to represent the alloantibody titre. Results shown are the reciprocal of the alloantibody titre obtained with □ anti-immunoglobulin; ▨ anti-IgG1; ▩ anti-IgG2.

(usually less than 1/32) and it is quite possible that this does not represent the major alloantibody response. Further studies with different assay systems are required to determine whether there is a significant non-complement-fixing antibody response as in the mouse.

The factors responsible for regulation of the type of antibody response have not been fully defined, although the nature of the antigen and the route of immunization are certainly major determinants. In allogeneic murine pregnancy much information is available on the ontogeny and tissue distribution of H-2 antigens on the fetus and placenta¹¹, but the precise source of the stimulatory antigen and the timing and route of maternal immunization are unknown. If the factors that favour a predominantly non-complement-fixing IgG1 antibody response could be determined, this would throw light on the mechanisms responsible for the survival of the fetus as an intra-uterine allograft, especially those directly implicating maternal alloantibody or antigen-antibody complexes in the blocking of cell-mediated immune reactions. The present findings also caution

against the use of experimentally hyperimmunized female mice in studies on the role of pregnancy-induced maternal alloantibody in other fetomaternal immunological interactions.

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Spontaneous remission of autoimmune encephalomyelitis is inhibited by splenectomy, thymectomy or ageing

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Experimental autoimmune encephalomyelitis (EAE) can be induced in genetically susceptible animals by injecting them with basic protein of myelin (BP) in a suitable adjuvant¹. EAE in adult Lewis rats is expressed clinically by acute paralysis and histologically by mononuclear cell infiltration of the central nervous system. Most rats spontaneously recover from EAE and show little or no damage to myelin². We report here that chronic progressive EAE with marked myelin lesions can be induced by a single injection of BP in complete Freund's adjuvant in intact 13-month old rats, or in 4-month old rats provided they have been splenectomized. Juvenile 2½-month old rats recover spontaneously despite splenectomy. Thymectomy of young adult rats leads to relapsing EAE. These results illustrate that the clinical course of EAE is markedly influenced by age and integrity of immune organs. Furthermore, they provide an experimental model with features similar to those of chronic relapsing disease of the nervous system of man.

Eleven to twelve days following a single injection of BP emulsified in complete Freund's adjuvant, young adult Lewis rats develop paralysis, primarily of the tail and hind legs. Histological examination of the brain typically shows perivascular infiltration of mononuclear cells. The myelin is usually intact, or only slightly damaged, and within 5–6 days most rats spontaneously recover. A proportion of female rats has been reported to relapse after recovery³ and the incidence of relapses can be increased by adrenalectomy after the onset of EAE⁴. Past experience² shows that relapses of EAE in young rats are not frequent and chronic EAE is rare.

EAE has been proposed as a model of human demyelinating diseases, such as multiple sclerosis⁵ but this condition is characterized by a relapsing clinical course and extensive damage to myelin. Therefore rat EAE, as an acute monophasic disease, with little evidence of demyelination, is not a very faithful model of multiple sclerosis. The mechanisms responsible for recovery from EAE have not been identified. The thymus and spleen are organs which function in regulating some aspects of immune reactions^{6,7}. To investigate the possible role of these organs in recovery we surgically removed the thymus from 6-week old Lewis rats and splenectomized 3-month old rats. When these rats were 4 months old we injected them with BP in adjuvant.

Untreated rats, or controls that underwent sham thymectomy or sham splenectomy, all developed EAE, judged by overt paralysis (see Table 1), and 10% of the rats died acutely. The remaining 90% were kept alive by individual supportive care with food and water, and all had a full clinical recovery. Histological examination of the brains 32 days after induction of EAE revealed perivascular infiltration of mononuclear cells. There was no evidence of myelin damage in these rats.

Four month old rats which had undergone thymectomy before induction of EAE demonstrated a different type of disease. Of the eight rats which survived the acute onset of EAE, three showed persistent paralysis. The other five recovered at about day 17, but all showed a relapse of clinical paralysis 3 days later, on day 20. Four of these five rats recovered from this relapse on day 24 but one had persistent paralysis. All the rats had marked perivascular infiltrates and all showed severe disruption of myelin.

Rats which had been splenectomized before induction of EAE developed acute disease, from which 2 out of 10 died. The remaining eight rats did not recover and showed persistent paralysis of the hind limbs until they were killed on day 32. The brains showed severe perivascular infiltration and myelin damage. Rats which had been thymectomized at 6 weeks then splenectomized at 3 months behaved about the same as those which had been splenectomized but not thymectomized.

It has been reported that spontaneous relapses are more common in 6-month old than in 3-month old rats⁸. Table 2 shows the results of an experiment in which we compared the responses of groups of rats which were splenectomized at 1½, 3 or 12 months. EAE was induced 1 month later. Rats aged 2½ months that had not died of acute EAE recovered, and no

Table 1 Chronic or relapsing EAE in 4-month old Lewis rats following splenectomy or thymectomy

Treatment	Day of onset	Acute EAE		Recovery		Chronic EAE	Relapse		Recovery from relapse		Grade of pathology
		(Total)	(Mortality)	Day	Incidence		Day	Incidence	Day	Incidence	
None	12	40/40	4/40	18	36/36	0/36	—	0/36	—	—	++
Sham											
thymectomy	12	10/10	1/10	16	9/9	0/9	—	0/9	—	—	++
Thymectomy	11	10/10	2/10	17	5/8	3/8	20	5/5	24	4/5	++++
Sham											
splenectomy	11	10/10	1/10	17	9/9	0/9	—	0/9	—	—	++
Splenectomy	10	10/10	2/10	—	0/8	8/8	—	—	—	—	++++
Thymectomy											
+splenectomy	10	10/10	3/10	—	0/7	7/7	—	—	—	—	++++

Thymectomy and splenectomy of female Lewis rats were performed under Nembutal anaesthesia (Veterinary Nembutal; Abbot), 60 mg per kg body weight. Thymectomies were done when rats were 6 weeks old and splenectomies when they were 3 months old. The animals were injected to induce EAE at the age of 4 months. Basic protein (500 µg ml⁻¹) extracted from guinea pig spinal cords was emulsified with an equal volume of Freund's adjuvant containing 4 mg ml⁻¹ H37Ra *Mycobacterium tuberculosis* (Difco). Each rat was injected with a total volume of 0.1 ml emulsion, containing 25 µg of basic protein, equally divided between the two hind footpads. The animals were examined daily for unequivocal signs of EAE which we considered to be overt paralysis of the tail and hind legs. Chronic EAE refers to paralysis that was unremitting from its onset until the experiment was terminated 32 days after injection. The surviving rats were killed and their brains and segments of spinal cord fixed for histological examination in 10% formalin, processed and embedded in paraffin for staining by haematoxylin and eosin, or by Luxol fast blue–periodic acid Schiff, to detect myelin damage. Pathology was scored on a scale + to +++++. (++++ Indicates disruption of myelin and severe mononuclear cell infiltrates in perivascular and periventricular areas, and in the parenchyma of the nervous tissue: spinal cord, brain stem, cerebellum and cerebrum. +++ Indicates no damage to myelin but severe and extensive infiltration as above. ++ Indicates moderate infiltration limited to perivascular areas with a spotty distribution. + Indicates mild perivascular infiltrates observed in at least one area.)

Table 2 Chronic or relapsing EAE as a function of age or splenectomy

Age (months)	Treatment	Day of onset	Acute EAE Incidence		Recovery		Chronic EAE Incidence	Relapse		Recovery from relapse Incidence	Grade of pathology
			(Total)	(Mortality)	Day	Incidence		Day	Incidence		
2½	None	11	8/8	2/8	17	6/6	0/6	—	0/6	—	++
	Sham splenectomy	12	8/8	2/8	16	6/6	0/6	—	0/6	—	++
4	Splenectomy	11	8/8	3/8	17	5/5	0/5	—	0/5	—	++
	None	12	8/8	1/8	17	7/7	0/7	—	0/7	—	++
	Sham splenectomy	12	10/10	1/10	17	9/9	0/9	—	0/9	—	++
	Splenectomy	11	10/10	2/10	—	0/8	8/8	—	—	—	+++
13	None	11	10/10	3/10	20	2/7	5/7	24	2/2	0/2	++++
	Sham splenectomy	12	10/10	4/10	19	2/6	4/6	24	2/2	0/2	++++
	Splenectomy	11	10/10	4/10	—	0/6	6/6	—	—	—	++++

Splenectomy of female Lewis rats was carried out at 1½, 3 or 12 months of age and EAE was induced 1 month later, as described in the legend to Table 1. Each age group was studied in a separate experiment. Repeat experiments involving rats of these ages all produced essentially the same results.

relapses were observed, whether or not the rats had been splenectomized a month earlier. Untreated or sham splenectomized 4-month old rats all developed acute EAE with about 10% mortality. All the surviving rats recovered without relapsing and their brains showed perivascular infiltrates with no damage to myelin. Similar to results shown in Table 1, the splenectomized rats that survived acute EAE all developed chronic paralysis and showed disruption of myelin on histological examination.

The 13-month old Lewis rats that were untreated or sham-splenectomized showed a relapsing or chronic course of EAE. Of the 13 rats that survived acute EAE in both of these groups 9 had unremitting, chronic paralysis. The four rats that recovered all suffered a relapse from which none recovered. Thirteen month old rats that had been splenectomized developed a chronic form of EAE similar to that of 3-month old splenectomized rats. These all had severe perivascular infiltrates and myelin damage.

EAE was also induced in unsplenectomized 15-month old rats. Of 14 rats inoculated, 5 died with acute EAE and 9 had chronic unremitting paralysis of the hind legs for 45 days, at which time the experiment was terminated.

It has been reported that male Lewis rats are more resistant to relapse of EAE than females³, but we have not yet had the opportunity to study the influence of sex on the effects of age or splenectomy.

In contrast to EAE in rats, EAE induced in guinea pigs is usually acutely fatal. However, injection of very young (2–4-week old) guinea pigs with guinea pig spinal cord in adjuvant produced a chronic or relapsing form of EAE after a relatively long latent period^{9,10}. Although there are differences between the rat and guinea pig models of EAE, these results emphasize the importance of an animal's age to the clinical course of EAE. The mechanisms by which age affects expression of EAE are probably complex, because age modifies the functions of organ systems which influence EAE, such as the immune and endocrine systems. It is conceivable that changes in the expression of EAE with age are related to changes in the function of the spleen.

Age also seems to have an important influence on the outcome of thymectomy. As we have shown (Table 1), thymectomy of 4-month old rats increases the severity of EAE. This contrasts with the effect of neonatal thymectomy, which has been observed to decrease markedly susceptibility to induction of EAE¹¹.

The effects of thymectomy and splenectomy indicate the importance of immune physiology in the clinical course of EAE. We have found (in preparation) that recovery from acute EAE is associated with suppression, in the draining lymph nodes, of the

proliferative response of T lymphocytes to a particular determinant of BP. The reactivity to other determinants on the BP molecule is undiminished during recovery. This suggests that the spleen and thymus may be involved in the physiological suppression of the immune response to a critical determinant on BP. However, splenectomy of 1½-month old rats failed to interfere with recovery from EAE induced a month later (Table 2). We have also observed that splenectomy of 3-month old rats 1 week rather than 4 weeks before induction of EAE did not prevent recovery from acute EAE (data not shown). Therefore, the task of the spleen is modified by the age of the subject and the timing of EAE induction. This suggests that the populations of cells mediating recovery are not always resident in or dependent on the spleen.

Our results suggest that defects in regulation of an immune response can transform what might otherwise be a self-limited insult into a chronic or relapsing disease of the nervous system. It is conceivable that multiple sclerosis or other progressive or relapsing diseases could be triggered acutely by a virus or some other noxious agent and be perpetuated by a defect in immune regulation. Subacute sclerosing panencephalitis, a chronic degeneration of the brain associated with infection by measles virus, has been suggested as an example of diseases involving irregularity of the immune response¹². It is possible that our experimental models of chronic or relapsing EAE might provide a means of identifying immune mechanisms regulating recovery from an autoimmune process and thus, a useful experimental approach to certain clinical problems such as multiple sclerosis.

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Precursor and effector phenotypes of activated human T lymphocytes

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In mice, thymus-derived lymphocytes are differentiated into functional subclasses by their cell surface antigens. The Ly 1 determinants are present on T cells with a helper function, whereas Ly 2 and Ly 3 antigens are expressed on the surface of lymphocytes with suppressor or cytotoxic functions¹⁻⁶. In man also, T-cell subsets have been identified using allo- and heteroimmune sera and, more recently, using monoclonal antibodies, which seem to identify helper and suppressor or cytotoxic subpopulations⁷⁻⁹. The major histocompatibility system (MHS)-encoded Ia antigens belong to several polymorphic families of membrane associated glycoproteins originally found on B lymphocytes^{10,11}; however, they have also been shown to be markers for suppressor T cells in mice^{12,13}. Recent studies have shown that in both mouse and man, T cells activated by a mixed lymphocyte reaction or by mitogens become Ia+¹⁴⁻¹⁸. Furthermore, some human T lymphoid cells, either freshly isolated from peripheral blood or after *in vitro* activation by lectins¹⁹⁻²¹ or alloantigens²²⁻²⁴, possess suppressor properties. We report here the phenotype of a T suppressor-cell subpopulation which was induced in long-term culture of lymphoid cells after activation with phytohaemagglutinin (PHA). Our results suggest that a subset of T cells was progressively expanded over a period of 8 days in culture and that, with the expression on the surface of these cells of 'Ia-like' antigens, they acquired the capacity to suppress the proliferative response of syngeneic or allogeneic lymphocytes to alloantigens or mitogens.

Peripheral blood lymphocytes (PBL) from six healthy donors were stimulated *in vitro* with PHA. The cultures were maintained for 30 days by repeatedly changing the culture medium. After 3, 8, 11, 18 and 23 days, 5×10^6 activated cells were taken from the suspension cultures and divided into two aliquots—one for assaying the suppressor activity in culture experiments, and the other for serological investigation of surface antigens on the activated cells.

To study their influence on syngeneic cells, activated cells were irradiated and mixed with equal numbers of syngeneic lymphocytes. Three of these mixed syngeneic/activated cell (SAC) cultures gave similar kinetic patterns with an exponential decline in stimulation: in donor V.A., SACs dropped to background stimulation by day 11, and in donors R.H. and J.A. by day 18 (Table 1). These results suggest that the decline of DNA synthesis in the SACs could be due to the development of suppressor cells. To test this, activated cells were added to cultures of normal lymphocytes stimulated either by purified protein derivative (PPD) or alloantigens. In SACs, reactions were prominent up to day 11, so that potential suppressor activity by J.A.- and R.H.-irradiated peripheral activated lymphocytes (IPALs) on PPD transformation could only be tested with '18-day' IPALs. Syngeneic PBL cultures mixed with '18-day' IPALs then stimulated with PPD were clearly suppressed by R.H., J.A. and V.A., but the suppressor V.A. IPALs were already present on day 8 after activation (Table 1).

Results from mixed lymphocyte culture (MLC) experiments designed to test the suppressor properties of '18-day' (experiment 1) and '23-day' (experiment 2) IPALs are shown in Table 2. These experiments have been repeated several times using frozen cells from '23-day' IPALs, with similar results. The suppressor effects of the IPALs were effective on syngeneic

R.H. and V.A., and also on allogeneic normal lymphocytes (LF).

The resting cells from three different donors were typed for the expression of DR antigens using the method of van Rood *et al.*²⁵, and the PHA-activated cells were typed using acridine orange and ethidium bromide at the end of the incubation period. Typings were done using alloantisera from the 7th and 8th International Workshops. Activated T cells were negative for DR antigens when tested on days 3 and 4 after activation, but were positive on day 8. In agreement with a previous report¹⁵, they expressed the same DR specificities present on the B cells of their resting lymphocytes. When the number of Ia+ cells was scored using two different anti-Ia monoclonal antibodies (McAB)—DA2 and 62-3-34 (ref. 26)—the number of donor RH Ia+ cells dropped from 28% on day 0 to become negative on day 3, and from day 8 progressively expanded to 83% on day 23 (Table 3). Similar patterns were observed with donors N.Y. (65% Ia+) and A.A. (51% Ia+).

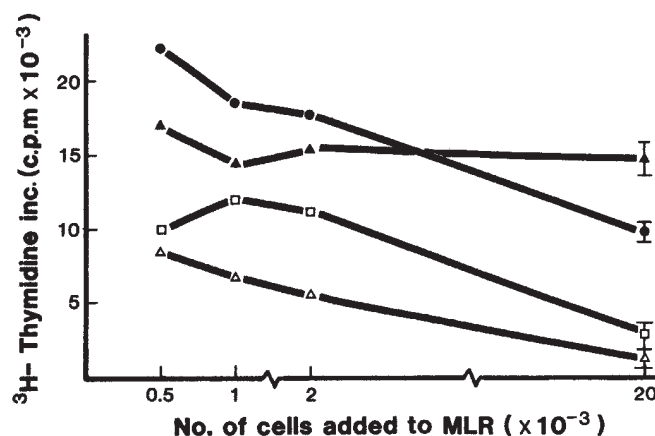


Fig. 1 Suppressor effect on an MLC by different cell numbers of an activated T cells subset. 20×10^7 day 23 activated cells were treated with 1:25 (final dilution) of DA2 for 30 min at room temperature, washed twice and incubated with 1:20 dilution of a rabbit anti-mouse FITC conjugated IgG. The cells were fractionated into weakly reactive (the lower 20% of fluorescence Ia-) and strongly reactive (the upper 60% of fluorescence Ia+) in a fluorescence-activated cell sorter (FACS-1). The MLC was carried out as described in the legend to Table 2. RH was used as responder and irradiated LF as stimulator. Different concentrations of irradiated normal syngeneic cells (▲), Ia+ (□), Ia- (●) or total unfractionated activated cells (Δ) were added to the MLC.

The activated cells were then typed for the expression of the TH2 marker, which had previously been reported to react with a small subset of T peripheral blood lymphocytes with suppressor properties⁸. The number of TH2+ cells from the responder N.Y. increased from 31% for the resting cells to 68% on day 23 after activation; cells from responder A.A. increased from 20% to 51% and R.H. from 17% to 71%. It was then important to establish if the expanded TH+ population also expressed the Ia antigens. The activated and resting cells were tested with two fluorescent labels for the simultaneous expression of Ia and TH2 (see Table 4). On day 11, 41 out of 45 (91%) R.H. TH2 T cells expressed the Ia antigen, compared with 12 out of 41 'day 7' A.A. cells (28%).

In another series of experiments, 7- and 23-day activated cells were separated into Ia+ and Ia- by means of a fluorescence activated cell sorter (FACS-1) with an anti HLA-DR (non-polymorphic) monoclonal antibody (DA2). The 7-day unseparated population contained 36% TH2+ cells and 12% Ia+. When separated into subpopulations, the Ia+ cells were

Table 1 Time course response to PPD of Ly from normal donors in presence of syngeneic irradiated normal or activated lymphocytes

		Day 3		Day 8	
	PPD	Normal cells	Activated cells	Normal cells	Activated cells
R.H.	-	8,070 ± 1,415	116,426 ± 4,637	1,390 ± 907	45,600 ± 3,215
R.H.	+	20,429 ± 1,571	170,569 ± 8,144	7,245 ± 1,640	30,684 ± 293
J.A.	-	3,123 ± 1,397	115,578 ± 10,963	1,390 ± 907	123,990 ± 9,090
J.A.	+	9,027 ± 1,733	118,481 ± 2,962	6,668 ± 2,292	108,069 ± 7,113
V.A.	-	5,175 ± 1,694	108,814 ± 18,665	3,489 ± 1,128	11,580 ± 6,282
V.A.	+	31,813 ± 9,990	74,334 ± 10,738	12,173 ± 611	7,055 ± 894

		Day 11		Day 18		
	PPD	Normal cells	Activated cells	Normal cells	Activated cells	% Inhibition
R.H.	-	2,007 ± 647	31,109 ± 7,585	5,141 ± 22	5,321 ± 558	
R.H.	+	7,174 ± 1,586	17,558 ± 6,388	15,408 ± 2,505	7,862 ± 2,277	(76%)
J.A.	-	3,501 ± 3,263	55,433 ± 3,160	2,607 ± 1,103	5,197 ± 826	
J.A.	+	9,944 ± 3,838	34,363 ± 7,818	12,312 ± 4,026	6,584 ± 1,286	(86%)
V.A.	-	4,703 ± 1,576	3,429 ± 2,094	4,762 ± 1,690	5,237 ± 1,719	
V.A.	+	12,358 ± 2,801	6,176 ± 1,507 (36%)	18,105 ± 3,176	7,895 ± 1,942	(81%)

PBL from donors R.H., J.A. and V.A. were stimulated in culture (1×10^6 cells per ml) with 1:100 final dilution of PHA for 36 h. The supernatant (conditioned medium, CM) was decanted and the cells resuspended in fresh medium (RPMI 1640 plus 10% AB serum) containing 30% CM (v/v final dilution). The cultures were maintained for up to 30 days by changing the medium with CM every 3-4 days. The suppressor effect of the activated T cells (>95% E-rosetting positive cells) on the response of resting cells to PPD was studied as follows: At 3, 8, 11 and 18 days after PHA activation, 5×10^4 activated cells in 50 μ l of medium (without CM) were irradiated with 4,000 rad and mixed with 5×10^4 syngeneic resting lymphocytes (50 μ l) in the presence or absence of 0.6 μ g of PPD (50 μ l). As a control, 5×10^4 resting cells were mixed with an equal number of syngeneic irradiated resting cells, in the presence or absence of PPD. Kinetic studies of DNA synthesis measured by the addition of 3 H-thymidine between days 3 and 6 showed that the peak of incorporation of isotope was on day 4.5. 3 H-thymidine uptake was measured on day 4.5 and percentage inhibition calculated as follows:

$$1 - \frac{\text{c.p.m. SAC(with PPD)} - \text{c.p.m. SAC(without PPD)}}{\text{c.p.m. resting cells(with PPD)} - \text{c.p.m. resting cells(without PPD)}} \times 100$$

Table 2 Suppressor capacity of activated T cells in MLC

		Stimulator cells*						
HLA phenotype Normal responder (50,000 cells per well)		Allogeneic cells (50,000 cells per well) L.F. K.H.†		Normal autologous (20,000 cells per well	Activated cells (20,000 cells per well) R.H. V.A.		c.p.m.	
Experiment 1								
Donor	R.H.	A1, AX/B8, BX/DR3, DR7	+		+	-	-	57,813±6,065
			+		-	+	-	3,111±817
			-		+	-	-	8,536±2,026
	V.A.	A2, A3/B35, B44/DR1, DR2	+		+	-	-	20,523±2,048
			+		-	-	+	2,229±738
			-		+	-	-	2,363±443
Experiment 2								
R.H.	A1, AX/B8, BX/DR3, DR7	+		+	-		25,718±4,296	
		+		-	+		2,158±185	
		-		+	-		6,619±514	
L.F.	A26, AX,/B38, BX/DR4, DR5	-	+	+	-		27,930±1,623	
			+	-	+		3,240±1,097	
			-	+	-		2,370±2,132	

50 μ l of autologous PBL (5×10^4) were cultured in quadruplicate on microtitre V-well plates in RPMI 1640 plus 10% AB serum, then 2×10^4 irradiated autologous normal PBL or '23 day' activated cells were added in 50 μ l of the same medium. 5×10^4 allogeneic cells were added to each well of this mixture. For each experiment, the cultures were pulsed for 16 h with 1 μ Ci of 3 H-thymidine after 2, 3, 4 and 5 days of culture, and results expressed in c.p.m. on the peak stimulation on day 5.5.

* Irradiated.

† HLA phenotype = A3,A11/B12,B35/DR2,DR4.

48% and the Ia⁻ cells were 32% TH2⁺. The 23-day activated population contained >90% Ia⁺ TH2⁺ cells.

Subsequently, the populations (Ia⁺ and Ia⁻) from 7- and 23-day activated cells were assayed for suppressor properties. With 23-day activated cells, most of the suppressor effect was confined to the Ia⁺ population (Fig. 1). However, when irradiated Ia⁺ or Ia⁻ 7-day activated cells were added to syngeneic responders and stimulated by a third party, both Ia⁺ and Ia⁻ cells stimulated strongly compared with controls (the cultures were assayed on day 4.5). These results suggest a carry-over of PHA although a helper effect of the activating cells cannot be ruled out completely, especially since 50% of the Ia⁺ population is not TH2⁺. Because both Ia⁺ and Ia⁻ populations were stimulators, no conclusions can be drawn about the relative suppressor properties of the various cell subpopulations in these 7-day cultures.

Table 3 Kinetic expression of TH2 and Ia antigens on activated T cells

Donor	Days after activation	TH2	Anti-Ia
N.Y.	0	31%	17%
	14	50%	42%
	22	68%	65%
A.A.	0	20%	15%
	7	42%	16%
	14	45%	48%
	22	48%	51%
R.H.	0	17%	28%
	4	30%	<1%
	9	40%	42%
	14	55%	51%
	22	71%	83%

Indirect immunofluorescence was used to analyse cell surface markers. Resting or activated cells (1×10^6) were treated with 1:50 (final dilution) of horse anti-human TH2 and with two anti-Ia McAB (DA2 and 62-3-34, both mouse anti HLA-DR non-polymorphic antibodies) at 1:25 (final dilution). The TH2 antibody was obtained from G. Janossy who prepared it from a batch of ATG (lot no. 17923, Upjohn) which was originally shown to have anti TH2 activity, and purified it according to the method of Reinherz and Schlossman⁸. The specificity was validated by comparing the prepared antibody with the Reinherz and Schlossman antibody on lymphocytes from a panel of 26 unrelated individuals and the reactions of the two antibodies were in complete agreement. After 20 min of incubation, 1:25 swan anti-horse FITC-labelled or 1:10 goat anti-mouse rhodamine conjugate antibodies were added to the second layer. Observations were made using a standard 14 Zeiss photomicroscope with epi-illumination (iv F1 epi-fluorescence condenser) and 2F1 reflector sleeve, equipped with exciter and barrier filters, for selective excitation and observation of fluorescence and rhodamine.

The changing antigenic profile of the activated T lymphocytes from Ia⁻ into Ia⁺ in a population of TH2⁺ cells is in sharp contrast to the profile of the resting population, in which there are less than 1% of TH2⁺ cells which are also Ia⁺. This implies either that this small sub-population had been expanded over 7 days of culture, or alternatively that the TH2⁺ Ia⁻ population, already expanded on day 4, became Ia⁺ after 8 days of culture. These data strongly suggest that cells with this phenotype are the active suppressors, although there is no direct evidence for this. Ideally one would like to be able to separate the cells into Ia⁺ TH2⁺ and Ia⁻ TH2⁺ cells, but the 23-day cells are practically all Ia⁺, whereas the 7-day cultures are all stimulatory, therefore such experiments are uninformative.

These findings may also have functional implications and may be important in the pathogenesis of certain diseases. For example, the increase of the Ia⁺ T cells *in vivo* could reflect the efforts

of the immune system to control the autoimmune reaction, for example in rheumatoid arthritis and systemic lupus erythematosus, where a disproportionate increase of Ia⁺ T cells in the peripheral blood has been reported²⁷. These alloantigens could be central to immunoregulation, and changing proportions of cells carrying one kind of alloantigen or another following stimulation by microorganisms or tumour-associated transplantation antigens may result in the production of suppressor/helper sub-populations.

Table 4 Simultaneous expression of TH2 and Ia⁺ antigens on the surface of PHA-activated cells

Responder	Days after activation	TH2 ⁺	Ia ⁺	Ia ⁺ and TH2 ⁺
A.A.	0	20%	15%	1%
A.A.	7	42%	16%	12%
R.H.	0	17%	38%	1.7%
R.H.	11	45%	53%	41%

2 × 2 contingency table:						
Responder	Days after activation	++	+-	-+	--	χ^2
A.A.	0	1	19	15	65	-3.39
	7	12	26	4	58	9.27
R.H.	0	2	16	36	46	-8.20
	11	41	4	12	43	44.90

See legend to Table 3 for experimental details. The contingency table shows number of TH2 positive versus number of monoclonal Ia positive cells.

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Monoclonal antibodies which distinguish between human NK cells and cytotoxic T lymphocytes

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Although it is widely accepted that T cells have a major role in specific tumour immunity, there is now much evidence that natural killer (NK) cells, which exist in many species and spontaneously lyse certain tumour cells *in vitro*, provide early resistance against tumour growth¹⁻⁴. Human NK-cell activity can be augmented *in vitro* by interferon⁵⁻⁷ and its inducers, including polyinosinic:polycytidylic acid (poly I:C)⁸; furthermore, NK-like activity is generated in mixed leukocyte cultures (MLCs) as is specific cytotoxic T-cell (T_c) activity⁹⁻¹¹. When effector cells generated in human MLCs lyse allogeneic or autologous virus-transformed or tumour cells⁹⁻¹³, it has been difficult to evaluate the relative contributions of T_c and NK-like cells to the lysis because the latter, like T cells, can form rosettes with sheep erythrocytes¹⁴ and react with xenogeneic anti-human thymocyte serum¹⁵. We report here that monoclonal antibodies against human mononuclear cell subpopulations can distinguish T_c from NK and NK-like cells. OKT3 or OKT8 monoclonal antibodies (reactive with virtually all or a subset of T cells, respectively^{16,17}) with complement (C') ablate MLC-generated T_c activity against allogeneic normal cells but do not decrease lysis of HLA-negative, NK-sensitive K562 leukaemia cells by NK, poly I:C-activated NK or MLC-activated NK-like cells. In contrast, OKM1 monoclonal antibody (reactive with a low proportion of non-adherent mononuclear cells as well as macrophages¹⁸) with C' causes marked diminution of NK and poly I:C-activated NK-cell activity.

The production and characterization of the monoclonal antibodies used in this study have been reported elsewhere¹⁶⁻¹⁹; all are ascites fluid preparations from mice inoculated with hybridoma cells producing antibodies of the IgG2 subclass, which fix rabbit C'. However, it had not been determined which, if any, of the antibodies with C' would eliminate cytotoxic effector cells. Thus, peripheral blood mononuclear cells, stimulated in MLC for 7 days with X-ray irradiated normal cells pooled from 20 unrelated individuals (pool_x), were treated with anti-human T-cell antibodies OKT3, OKT8, OKT4 or control ascites fluid, followed by the addition of C', as detailed in Table 1 legend. Cytotoxic activities of the treated effector cell populations were compared against ⁵¹Cr-labelled allogeneic normal lymphocyte targets which are lysed by T_c but not by fresh or activated NK cells⁷. Typical results of one of five experiments, shown in Table 1, indicate that treatment of MLC-generated effector cells with OKT3 or OKT8 and C' totally ablated T_c activity against the normal allogeneic cells. OKT4 and C' did not decrease T_c activity; however, it is unclear why enrichment of cytotoxicity was not observed following removal of the 33% cells killed by this antibody, because previous results, obtained using the fluorescence-activated cell sorter, showed that OKT4 reacts with a subset of T cells lacking T_c activity¹⁹.

Experiments were carried out to determine whether the monoclonal anti-T-cell antibodies would decrease either non-augmented or poly I:C-augmented NK-cell activity against

NK-sensitive K562 cells. Representative results (Fig. 1) confirm our previous report⁸ that poly I:C augments human NK-cell activity against K562 cells by approximately 2.5-fold (44.6 lytic units (LU) compared with 106 LU). Exposure of untreated or poly I:C-treated non-adherent mononuclear cells to OKT3 or OKT8 and C', which deplete T_c activity, did not decrease NK or poly I:C-augmented NK activity against K562 cells in this or in two similar experiments; additionally, OKT4 and C' failed to decrease NK-cell activity. The increase in lytic units per 10⁶ cells, following treatment with the anti-T-cell antibodies and C', can be accounted for primarily by enrichment for NK cells resulting from lysis of T cells; this is because the total LU recovered following antibody or control ascites treatments were similar, except for the less than twofold increase in LU recovered following treatment of poly I:C-activated cells with OKT3 and C' (Fig. 1b). In contrast, OKM1 and C' (which lysed 11-14% of the cells) reduced NK and poly I:C-augmented NK-cell activity against K562 cells by at least 75% in this and in two other experiments. It thus seems that the majority of NK and poly I:C-activated NK cells are OKM1⁺ and OKT3⁻,

Table 1 Depletion of MLC-generated T_c against allogeneic normal cells by monoclonal antibodies OKT3 and OKT8

Treatment of A pool _x effector cells	% Specific ⁵¹ Cr release from B ± s.d.	
	30:1	10:1
Control ascites + C'	56.8 ± 8.9	38.7 ± 7.5
OKT4 + C'	50.7 ± 8.4	37.2 ± 4.8
OKT8 + C'	3.7 ± 3.9	2.7 ± 3.6
OKT3 + C'	7.0 ± 4.3	5.6 ± 4.0

Ficoll-Hypaque-isolated mononuclear cells from individual A were washed and resuspended in culture medium consisting of RPMI 1640 (Gibco) supplemented with 25 mM HEPES buffer and 15% heat-inactivated normal human serum. 9 × 10⁶ responding cells from individual A were incubated in a final volume of 10 ml culture medium in Falcon T25 tissue culture flasks with 9 × 10⁶ X-ray irradiated (2,500R) allogeneic normal lymphocytes, pooled in equal numbers from 20 unrelated individuals (pool_x) as previously described¹². On day 7 after the onset of MLC, the non-adherent A pool_x effector cells were collected from flasks, washed once and treated in culture medium with control ascites from mice inoculated with the parental myeloma cell line or ascites fluid containing monoclonal antibodies OKT4, OKT8 or OKT3 (produced as described elsewhere¹⁶⁻¹⁹), at a final dilution of 1:100 for 1 h at 4°C. Rabbit complement (C') (Pel-Freez Biologicals) was added at a final dilution of 1:2. Following a 1-h incubation at 37°C, the % viable cells, determined by trypan blue dye exclusion, was 98, 67, 57 and 26% for cells treated with control ascites, OKT4, OKT8 and OKT3, respectively. The treated cells were layered on Ficoll-Hypaque gradients to remove dead cells and the viable cells isolated from the interfaces were washed and resuspended in complete medium before their use as effector cells. Effector cells were added to round-bottom wells of microtitre trays (0.8-2.4 × 10⁵ per well) followed by the addition to each well of 8 × 10³ ⁵¹Cr-labelled allogeneic normal lymphocytes from individual B; the resulting effector to target cell ratios were 30:1 and 10:1. Following a 7-h ⁵¹Cr-release assay at 37°C, a constant amount of supernatant was removed from each well and transferred to tubes for counting in a γ-counter. The % specific ⁵¹Cr release from target cells was calculated as follows:

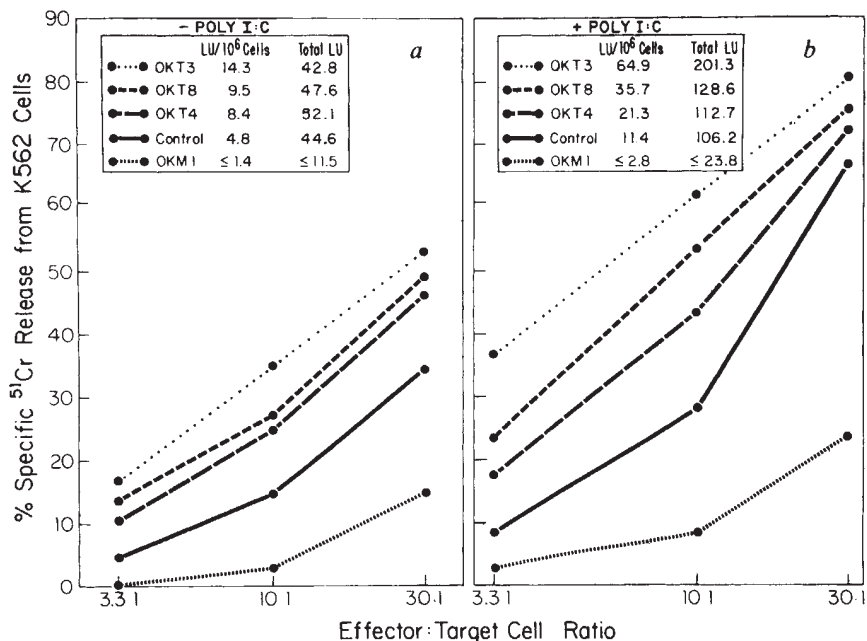
$$\frac{(\text{c.p.m. test release} - \text{c.p.m. spontaneous release})}{(\text{c.p.m. maximal release} - \text{c.p.m. spontaneous release})} \times 100$$

where spontaneous release = c.p.m. release from target cells in medium alone, and maximal release = c.p.m. release from target cells in detergent. Spontaneous ⁵¹Cr release was 13.2% of the maximal release. The values shown are the mean specific % ⁵¹Cr released from four replicate wells ± s.d. The % specific ⁵¹Cr released from the cells by mononuclear cells from individual A cultured for 7 days without stimulating cells was 4.2 ± 1.3%. The % specific ⁵¹Cr released from the target cells by A pool_x effector cells treated with control ascites and C', control ascites alone, C' alone or antibodies in the absence of C' did not differ from that released by untreated effector cells.

OKT4⁻ and OKT8⁻. This finding firmly supports a recent hypothesis that NK cells might be OKM1⁺, which was based on data showing that OKM1 antibody reacts in immunofluorescence assays with the majority of non-adherent cells having surface characteristics of NK cells²⁰, that is, receptors for sheep erythrocytes¹⁴ and for the Fc portion of IgG (refs 7, 11, 14). Our failure to ablate NK-cell activity totally by OKM1 and C' (Fig. 1) suggests that either a small percentage of NK cells are OKM1 or that they express a very low density of OKM1 antigen.

Effector cells generated in MLC can lyse certain allogeneic and autologous lymphoblastoid cell lines and leukaemia

Fig. 1 Effect of monoclonal antibodies on NK and poly I:C-augmented NK-cell activities. 10×10^6 Ficoll-Hypaque isolated mononuclear cells were cultured in Falcon T25 tissue cultured flasks containing 5 ml RPMI 1640 medium supplemented with 25 mM HEPES buffer, 15% normal human serum and 0 (a) or 100 μ g (b) poly I:C ml^{-1} . Following a 16-h incubation at 37°C, the non-adherent cells (containing <1% macrophages, as judged by nonspecific esterase staining) were collected and treated with antibodies or control ascites and C' as detailed in Table 1; OKM1 was used at a dilution of 1:10. The numbers of viable cells recovered after treating 10^7 cells with OKT3, OKT8, OKT4, control ascites or OKM1 and C' were 3.0, 5.0, 6.2, 9.3 or 8.2×10^6 , respectively (a), and 3.1, 3.6, 5.3, 9.3 or 8.5×10^6 , respectively (b). The washed effector cell populations were tested for cytotoxicity against ^{51}Cr -labelled K562 cells in a 5-h ^{51}Cr release assay. The data points represent the mean % specific ^{51}Cr released from K562 cells in four replicate wells, calculated as in Table 1; the s.d. values were <12% of the mean values. Spontaneous ^{51}Cr release was 11.2% of maximal release. 1 LU (lytic unit) was defined as the number of effector cells required to cause 35% specific ^{51}Cr release from 8×10^3 target cells. Total LU recovered after treating 10^7 cells was calculated as follows: LU per 10^6 cells $\times$ no. of viable cells recovered.

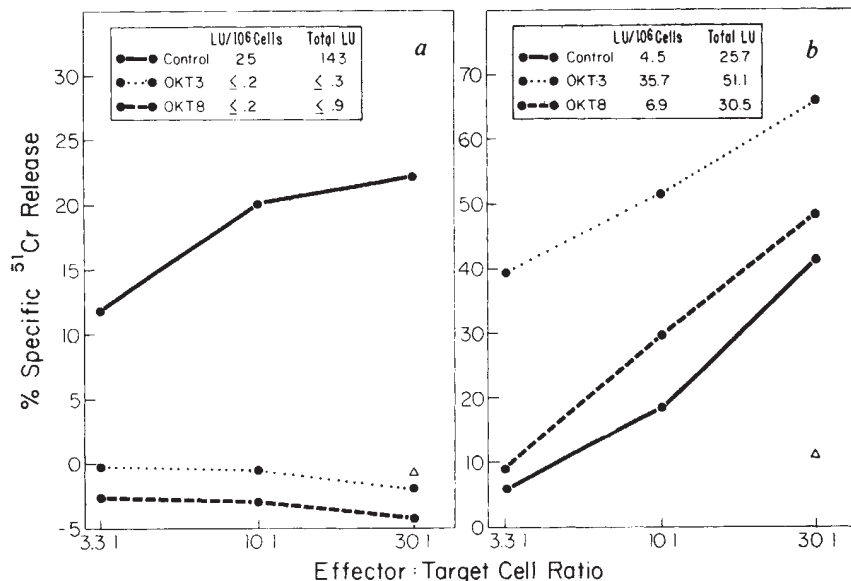


cells⁹⁻¹³, which may be sensitive to lysis by NK or activated NK cells⁷. It is difficult to determine the proportion of such cytotoxicity that is mediated by T_c or NK-like cells; the mere observation that NK-sensitive target cells are lysed is insufficient to exclude participation by T_c in the lytic reaction. In attempts to distinguish T_c from NK-like cells, effector cells generated in MLC by stimulation with pooled allogeneic normal cells, were treated with OKT3 or OKT8 and C' and were then tested for their ability to lyse allogeneic normal lymphocytes and HLA-negative K562 cells. Results shown in Fig. 2a, similar to those shown in Table 1, indicate that OKT3 or OKT8 plus C' depletes T_c activity against allogeneic normal lymphocytes; in contrast, treatment of the stimulated cells with OKT3 or OKT8 and C' did not decrease cytotoxicity against the NK-sensitive K562 leukaemia cells (Fig. 2b). Similar results were obtained in four other experiments; however, the total LU recovered against K562 cells from OKT3-treated cells was not consistently higher than that recovered from control ascites-treated cells. Our results thus demonstrate that T_c can be distinguished from MLC-activated NK-like cells by use of monoclonal OKT3 or OKT8 antibodies and C'.

Although we observed that OKM1 plus C' caused marked diminution of fresh and poly I:C-activated NK cells (Fig. 1), we have failed, with such a treatment, to eliminate cytotoxic activity of effector cells generated in MLC against allogeneic normal cells or K562 cells (data not shown). This finding is reminiscent of observations that NK as well as interferon- and poly I:C-augmented NK-cell activities are depleted by removing cells expressing receptors for the Fc portion of IgG (refs 7, 8, 11, 14); however, both NK-like cells and T_c generated in MLC seem to lack Fc receptors^{11,21}. The relationship between OKM1 antigen and the Fc receptor on NK cells remains to be elucidated. It is possible that MLC-activated NK-like cells are derived from OKM1⁺ cells but that following several days in MLCs, the OKM1 antigen may not continue to be expressed on these cells; alternatively, the NK-like cells generated in MLCs may originate from a phenotypically different precursor cell.

As monoclonal antibodies directed against human mononuclear cell subpopulations can distinguish T_c from NK and NK-like cells, it is now possible to delineate the effector cells that are cytotoxic for a variety of cell lines and autologous malignant cells.

Fig. 2 Distinction between T_c and NK-like cells generated in MLC by monoclonal anti-T-cell antibodies. Peripheral blood mononuclear cells were stimulated for 7 days in MLC with X-ray irradiated pooled allogeneic normal cells and collected and treated with monoclonal antibodies OKT3 or OKT8 and C' as detailed in Table 1 legend. The numbers of viable cells recovered after treating 10^7 cells with control ascites, OKT3 or OKT8 with C' were 5.7, 1.4 or 4.4×10^6 , respectively. The effector cells were then tested for cytotoxicity against allogeneic normal lymphocyte targets (a) and HLA-negative K562 leukaemia cells (b) in 7-h and 5-h ^{51}Cr release assays. The % specific ^{51}Cr released from targets by responding cells cultured in the absence of stimulating cells is designated by Δ . Negative % specific ^{51}Cr release values indicate that less ^{51}Cr was released from target cells in the presence of effector cells than that which was released spontaneously in medium alone. Spontaneous ^{51}Cr release from allogeneic normal cells and K562 cells was 14.8 and 10.2% of maximal ^{51}Cr release. LU per 10^6 cells and total LU recovered from 10^7 cells were calculated as shown in Fig. 1. One lytic unit was defined as the number of effector cells required to cause 15% or 40% specific ^{51}Cr release from 8×10^3 labelled allogeneic normal cells or K562 cells, respectively.



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H-2-linked genes control immune response to V-domains of myeloma protein 315

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Immunoglobulins function as antigen receptors of B lymphocytes and as effector molecules for elimination of foreign antigens. Recently, other functions have been revealed: for example, idiotypes (antigenic sites of variable (V) domains) bound on the surface of plasmacytomas^{1,2} can act as tumour-specific transplantation antigens³. Moreover, optimal maturation of B lymphocytes of a given idiomotype into antibody-secreting cells seems to require assistance from two different kinds of helper T cells (Th), one bearing receptors complementary or similar to that idiomotype, and another that recognizes the antigen⁴⁻⁶. To learn more about recognition of V regions by Th, we have used V-domains of the immunoglobulin A (IgA) ($\lambda 2$) myeloma protein M315 produced by the BALB/c plasmacytoma MOPC 315 (ref. 7) as carriers for anti-hapten responses. We report here that the Th response to the V-domain of the heavy chain (V_H) and the light chain (V_L) of M315 seems to be under H-2-linked immune response (IR) gene control.

Various strains of mice were injected separately with 100 μ g of V_H^{315} and V_L^{315} (carrier) and 200 μ g of hapten coupled to bovine serum albumin (BSA). The hapten was the (4-hydroxy-5-iodo-3-nitrophenyl)acetyl (NIP) group. The Th responses to the two carrier antigens were determined by transferring the carrier-primed spleen cells to irradiated (500R X-ray) syngeneic recipients together with hapten-primed B cells. We concluded that those strains in which a pronounced anti-NIP response was observed 10-12 days following boost with 200 μ g of NIP₃-Fab³¹⁵ in saline had responder Th, whereas those strains in which the anti-NIP response was very low had non-responding Th. Previous studies had established that the BALB/c anti-NIP antibody response in this adoptive transfer system is Th dependent^{8,9}. For example, BALB/c spleen cells primed with minimal essential medium (MEM) or BSA have never evoked a secondary anti-NIP response above 7.5% (see Table 1).

This analysis revealed that for each antigen the various mouse strains could be clearly distinguished into high responders and non-responders (Table 1). Thus, mice of the d (BALB/c and DBA/2), b (C57BL) and s (SJA, SJL) haplotypes made strong anti-NIP antibody responses when they received Th primed with V_L^{315} . Mice of the k (CBA, C3H and AKR) haplotype made no or poor anti-NIP responses. For unknown reasons, CBA mice from Gl. Bomholtgaard gave a higher response to V_L than the other H-2^k strains (Table 1, expt A), including CBA from the Jackson Laboratory (Table 1, expt B).

A different genetic pattern was observed for Th responses to V_H^{315} . Mice of the k and s haplotypes made strong anti-NIP responses, whereas mice of the b and d haplotypes made no or weak responses (Table 1). The Th of (C3H $\times$ BALB/c)F₁ mice responded to both V_H and V_L priming (Table 1), showing that the responder phenotypes are inherited in a dominant fashion.

Mice of the b (C57BL/6), d(BALB/c) and k (C3H, CBA, AKR) haplotypes were also primed with the Fv fragment of M315 which contains both the V_H and the V_L domains. In contrast to the distinct response patterns observed with the isolated V-domains, all strains were responders to Fv (Table 1, expt C). We have been unable to demonstrate suppressor activity in spleen cells of V_H^{315} -primed BALB/c mice. Thus, the anti-NIP responses of recipients that received a mixture of 20×10^6 V_L - and 80×10^6 V_H -primed spleen cells were equivalent to the responses of animals receiving only V_L -primed cells (data not shown).

The importance of the major histocompatibility complex (MHC) genes in these responses is shown by the correlation between MHC type and ability to respond to either V_H or V_L (Table 1). Furthermore, the congenic strain BALB.K (H-2^k), which only differs from BALB/c at the H-2 complex, responds like mice of the k haplotype (Table 2). Thus, it is a non-responder to V_L^{315} , although BALB/c responds, and it is a responder to V_H^{315} , although BALB/c does not respond. There was no correlation between Igh-C allotypes and response patterns (Table 1). These results provide strong evidence that distinct genes within the MHC control the Th responses to these V-domains.

What is the nature of the V-domain epitopes recognized by the Th of this study? The genetic background of the mouse in which MOPC-315 arose was the seventh generation of successive backcrosses to inbred BALB/c mice, starting with the F₁ progeny of a BALB/c $\times$ C57BL/Ka cross. The progeny selected for each backcross with BALB/c had both BALB/c and C57BL/Ka allotypic Igh-C markers¹⁰. As M315 bears the same Igh-C allotypes (A^{12,13,14}) as BALB/c IgA myeloma proteins⁷, the BALB/c origin of C_H³¹⁵ is established. It has not been possible to prove the BALB/c origin of L³¹⁵ because no allotypic markers have been found on the $\lambda 2$ chain. However, the probability that L³¹⁵ is the product of C57BL genes is only 1/256 (ref. 10). As both BALB/c and C57BL mice have on average 11-14 μ g per ml of protein immunochemically related to V_L^{315} (ref. 11) and are responders to V_L^{315} (this work), it is unlikely that the antigenic determinant is an allotype. Hence we conclude that it is related to idiotypes. For V_H^{315} one cannot rule out allotypy as both BALB/c and C57BL are non-responders.

These experiments demonstrate that complete immunoglobulins may be unsuitable for priming Th in studies of MHC-linked IR gene control of immune responses to V-regions because complete immunoglobulins may, as with M315, bear more than one genetically controlled V-region determinant. This is illustrated by the uniform high responsiveness of all strains to the Fv-315 fragment. In addition, new epitopes formed when V_H and V_L are assembled, as in Fv, may also contribute to these high responses. Therefore, the lesser antigenic complexity of individual domains offers certain advantages in the dissection of the immunogenicity of complete immunoglobulins. Second, a significant set of Th present in many mouse strains recognize the V-domains of M315 in another way than most B lymphocytes. Thus, the anti-idiotypic antibodies raised in isogenic mice against M315 usually bind to

Table 1 Separate genetic control of helper T-cell (Th) responses to V_H^{315} and V_L^{315}

Strain* immunized	H-2 type	Igh-C type	Secondary anti-NIP response in recipients of Th primed with:					
			V_H	Expt A	V_L	Expt B	V_L	Expt C Fv
BALB/c	d	a	3.4 (3.0)	64.3 (2.8)		0.0	56.4 (1.7)	67.7 (1.0)
DBA/2	d	c	5.6 (0.9)	60.8 (1.8)		4.8 (0.8)	29.6 (5.9)	
NZB	d	e	3.6 (1.3)	30.9 (1.5)				
C57BL†	b	b	7.7 (1.1)	33.3 (4.6)		0.9 (0.9)	34.4 (1.1)	51.3 (8.3)
C3H	k	a	39.9 (2.4)	7.9 (2.2)		52.5 (3.5)	8.0 (2.9)	68.3 (0.5)
CBA	k	a	52.6 (3.2)	22.8 (7.3)		49.4 (2.6)	1.5 (0.6)	59.0 (2.8)
AKR	k	d				52.7 (1.1)	5.1 (1.7)	80.8 (0.8)
(C3H×BALB/c)	k×d	a				41.1 (6.1)	32.7 (6.0)	
SJL	s	b	45.5 (5.2)	66.4 (2.0)		50.1 (3.7)	54.9 (5.5)	
SJA	s	a	26.1 (3.3)	43.2 (4.3)				

Each irradiated (500R X ray) recipient was given $\frac{1}{2}$ spleen equivalent of NIP-BSA- and carrier-primed cells⁹. The values represent per cent of 1.2×10^{-6} mol of $N-[^{125}I]$ P-caproic acid bound by 20 μ l serum collected 10 days (expts A and B) and 13 days (expt C) after boost with NIP₃-Fab³¹⁵. BALB/c spleen cells primed with MEM or BSA have never evoked a secondary anti-NIP response above 7.5% in this assay system. The numbers in parentheses are s.e.

* Mice of expts A and C were from Gl. Bomholtgaard, and of expt B from the Jackson Laboratory. The SJA strain was from Institute of Cancer Research, Philadelphia.

† C57BL/6 in expts A and C; C57BL/10 in expt B.

'neo' antigenic idiotype determinants that form when the V_H and V_L domains assemble¹². We propose that the Th primed with individual V^{315} domains encounter and recognize the same domains in an assembled form when the animals are boosted with NIP₃-Fab³¹⁵, and that these Th focus on immunogenic sites shared by free and assembled V-regions. According to this hypothesis each member of the M315 pair of V-domains contains sufficient structural information by itself to be recognized by Th. This interpretation is in line with Edelman's proposal that each of the compact domains of immunoglobulin chains has evolved to perform a special function¹³, and contrasts with the combined contribution of V_H and V_L domains to the construction of the antigen-binding sites of complete antibodies^{14,15} and many, but not all, serologically defined idiotypes.

The broader implications for the idiootype network¹⁶ concept are that the V-region-specific Th of the present study, detected by a conventional hapten-carrier system, may be envisaged to be able to operate directly on the immunoglobulin receptors of B lymphocytes like the idiootype-specific set of Th (refs 4–6). Hence, V_H and V_L may also function as independent targets in the idiootype network and the same Th may be able to perform both carrier- and idiootype-specific help.

An objection to this interpretation is that the immunoglobulin receptors of B cells presumably are not subject to antigen processing before recognition by idiootype-specific Th whereas such processing may have taken place with the antigens of the present study. Evidence against this objection derives from an earlier experiment where L^{315} priming of Th from BALB/c mice not only augmented the anti-NIP responses but also the antibody responses to the M315 idiootype of recipients boosted with M315 (ref. 8). As the serologically defined idiootype of M315 requires assembled ($V_H + V_L$) domains for its expression, and as the determinants recognized by collaborating B and Th cells must be present on the same molecular complex¹⁷, this result suggests that the V-domains of M315 used in the present

experiments were recognized as intact during priming and assembled during the boost.

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Sperm-specific surface antigenicity common to seven animal phyla

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While studying the plasma membrane of sperm of the sea urchin *Strongylocentrotus purpuratus*, we developed an antiserum that exhibits unusual reactivity—it cross-reacts with the surfaces of spermatozoa of 28 species representing seven phyla of the animal kingdom. A negative cross-reaction has not been found. This suggests the possible existence of common antigenic determinants on the surface of all animal sperm. We report these preliminary results here because of the broad implications common sperm-surface antigenicity has for the potential development of immunocontraceptive methods.

Table 2 T-helper cell recognition of V_H^{315} and V_L^{315} is regulated by genes within the MHC complex

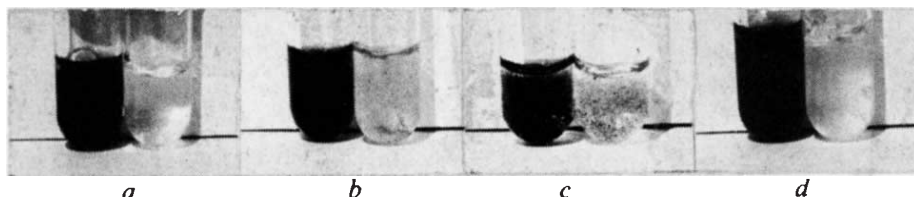
Strain* immunized	H-2 type	Secondary anti-NIP response in recipients of Th primed with:	
		V_H	V_L
BALB/c	d	5.9 (2.3)	57.8 (1.0)†
C3H	k	61.3 (0.6)	8.5 (1.1)
BALB.K	k	27.1 (2.9)	2.0 (0.2)

* The strains BALB/c and C3H were purchased from Gl. Bomholtgaard. BALB/K mice were from Olac 1976.

† Values expressed as in Table 1. The serum was collected 11 days after boost with NIP₃-Fab³¹⁵.

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Fig. 1 Cross-reactivity of SSA with sperm of four species visualized by the immunoperoxidase procedure. Approximately equal numbers of spermatozoa ($5 \times 10^8 \text{ ml}^{-1}$) were used for each reaction (tube on left, SSA; tube on right, preimmune serum). a, *S. purpuratus* (sea urchin); b, *Rattus norvegicus* (rat); c, *Salmo* sp. (salmon); d, *Metridium senile* (sea anemone).



fibriatum (sea anemone). To prepare SSA, sperm of the sea urchin *S. purpuratus* were washed by sedimentation (1,000 g, 10 min) in filtered sea water to remove seminal fluid, fixed 1 h in 3% glutaraldehyde-sea water, then washed to remove the fixative. They were resuspended in phosphate-buffered saline (PBS, pH 7.2, 0.5 mg per ml sperm protein), mixed with Freund's complete adjuvant (1 Freund's: 2 sperm) and a virgin female New Zealand rabbit was immunized by direct injection into the popliteal lymph nodes¹⁴. After 4 weeks a second injection was given subcutaneously and the rabbit bled 10 days later. Serum was removed following clot retraction and heated to 56 °C for 30 min. To eliminate non-sperm-specific activities, antiserum was absorbed with minced sea urchin ovary and non-gametic male tissues (coelomocytes and tube feet) by incubating the tissues in antiserum, then clarifying by centrifugation. To obtain a 1% TX-100 extract, sperm were washed and resuspended in sea water containing 10 mM TAME (*p*-tosyl-1-arginine-methyl ester) and 10% TX-100 in sea water added to a final concentration of 1%. After 30 min the soluble extract was collected by centrifugation at 30,000 g for 30 min. To perform the immunoperoxidase localization, sperm were fixed for 1 h in 3% glutaraldehyde- or formaldehyde-sea water (or PBS), washed and then incubated for 30 min in 10% normal swine serum (NSS). Following removal of excess NSS, the cells were incubated 1 h in preimmune serum (1/80) or SSA (1/80), washed three times in 1% NSS-PBS, incubated 15 min in horseradish peroxidase-conjugated swine anti-rabbit IgG (1/50; Bio-Rad Laboratories) and washed three times in 1% NSS-PBS over a 12-h period. The cells were then resuspended in 1 ml reaction mixture, containing 0.75 mg Hanks-Yates reagent (Polysciences), 0.1 M Tris-HCl, pH 7.6, and 0.01% H_2O_2 (ref. 15). After 15 min the cells were washed in PBS, transferred to a clean tube and the presence or absence of reaction product determined by direct visual observation or phase-contrast microscopic observation of the cell suspension. The precipitate was visible over the entire cell surface. No free precipitate was observed in the suspension. Preimmune serum did not react. Hanks-Yates reagent was used in place of diaminobenzidine because the latter is a potent carcinogen.

The antiserum was prepared using whole, glutaraldehyde-fixed *S. purpuratus* sperm as the immunogen. Fixed cells may be a better immunogen than their unfixed counterparts because they are not cleared by macrophages as readily as unfixed ones, thus creating an artificial booster effect¹. The antiserum to the fixed sperm produced several precipitin lines when diffused in agar against a Triton X-100 (TX-100) extract of sea urchin sperm. Following absorption of the whole antiserum with sea urchin ovary and non-gametic male tissues, only one precipitin line appeared in the agar plate. However, we recognize that minor antigenic activities may remain undetected by simple immunodiffusion assay (the number of antigenicities present in the absorbed serum is not the point of this letter). This absorbed antiserum is hereafter designated 'sperm-specific antiserum' (SSA). The sperm-specific activity of SSA could be completely eliminated by absorption with fixed or living sperm [as determined by immunodiffusion, immunoperoxidase and indirect immunofluorescence (IIF)]. The SSA inhibited the egg jelly-induced acrosome reaction in living *S. purpuratus* sperm.

When the SSA was tested for cross-reactivity (using immunoperoxidase and IIF) with sperm of other species, we found that every species gave a positive reaction (Figs 1 and 2, Table 1). Rat sperm (Figs 1b and 2d), salmon sperm (Fig. 1c) and coelenterate sperm (Fig. 1d) reacted as strongly as sea urchin sperm (Figs 1a and 2b). We stress that this is a qualitative assay and that we have not determined the number of binding sites for the SSA on a per cell basis. The results obtained with IIF (Fig. 2) and phase-contrast and bright-field observations of immunoperoxidase-reacted cells showed that the activity is distributed over the entire surface of the sperm. Controls on the immunoperoxidase procedure (Fig. 3) showed that the deposition of the coloured reaction product depended on the reaction of the SSA with the sperm surface. A variety of cell types were tested to determine that the activity was indeed sperm specific; no non-sperm cell types reacted with the SSA (Table 2).

The unusual reactivity of this antiserum led us to perform an extensive series of controls to establish that the sperm-specific activity was not artefact. Because glutaraldehyde-fixed sperm were used as the immunogen, we examined whether the activity of the SSA might be due to a glutaraldehyde-hapten effect. This has been ruled out by the following findings: (1) The SSA raised against the glutaraldehyde-fixed sea urchin sperm reacted with both living sperm and formaldehyde-fixed sperm. (2) Another antiserum, raised by injecting unfixed, whole sea urchin sperm into a rabbit, had the same reactivity as the serum to glutaraldehyde-fixed sperm. (3) A third antiserum produced by

using a 1% TX-100 extract of sea urchin sperm as the immunogen also had the same specificity. (4) The activity of the SSA was completely eliminated by absorbing the antiserum with either living or glutaraldehyde-fixed sperm, also showing that the activity was directed against surface molecules.

Because there are carbohydrate sequences in complete Freund's adjuvant that might elicit the production of antibodies that cross-react with some mammalian sperm², two experiments were performed to show that the activity of the SSA was not the result of this phenomenon. First, a rabbit was injected with complete Freund's adjuvant only from the same vial of adjuvant that had been used in production of the SSA. Both the pre-injection and post-injection sera from this rabbit showed no reactivity with sperm. Second, a rabbit was immunized with

Table 1 Reaction of SSA prepared against *S. purpuratus* sperm (sea urchin) with sperm of other species

PHYLUM COELENTERATA	PHYLUM ECHINODERMATA
Class Anthozoa	Class Echinoidea
<i>Metridium senile fibriatum</i>	<i>S. purpuratus</i>
PHYLUM ANNELIDA	<i>S. franciscanus</i>
Class Polychaeta	<i>S. pallidus</i>
Fam. Spionidae (1 species)	<i>S. droebachiensis</i>
Fam. Polynoidae (1 species)	<i>Lytechinus pictus</i>
PHYLUM MOLLUSCA	<i>Arbacia punctulata</i>
Class Amphineura	<i>Triplonectes gratilla</i>
<i>Cryptochiton stelleri</i>	<i>Dendroaster excentricus</i>
Class Gastropoda	Class Ophiuroidea
<i>Acmaea</i> sp.	<i>Ophioplocus esmarkii</i>
Class Pelecypoda	Class Asteroidea
<i>Macoma nasuta</i>	<i>Patiria miniata</i>
PHYLUM ECHIUROIDEA	PHYLUM CHORDATA
<i>Urechis caupo</i>	Class Urochordata
PHYLUM ARTHROPODA	<i>Styela clava</i>
Class Crustacea	<i>S. plicata</i>
<i>Cancer antennarius</i>	<i>Ciona intestinalis</i>
<i>Pinnixa tubicola</i>	Class Osteichthyes
Class Merostomata	<i>Salmo</i> sp.
<i>Limulus polyphemus</i>	Class Amphibia
	<i>Rana pipiens</i>
	Class Aves
	<i>Meleagris gallopavo</i>
	Class Mammalia
	<i>Mesocricetus auratus</i>
	<i>Rattus norvegicus</i>

Reactivity of the SSA was assayed by IIF or by the immunoperoxidase procedure. The sperm were fixed in either 3% glutaraldehyde or formaldehyde. Preimmune serum did not react. See Figs 1 and 2 legends for methods.

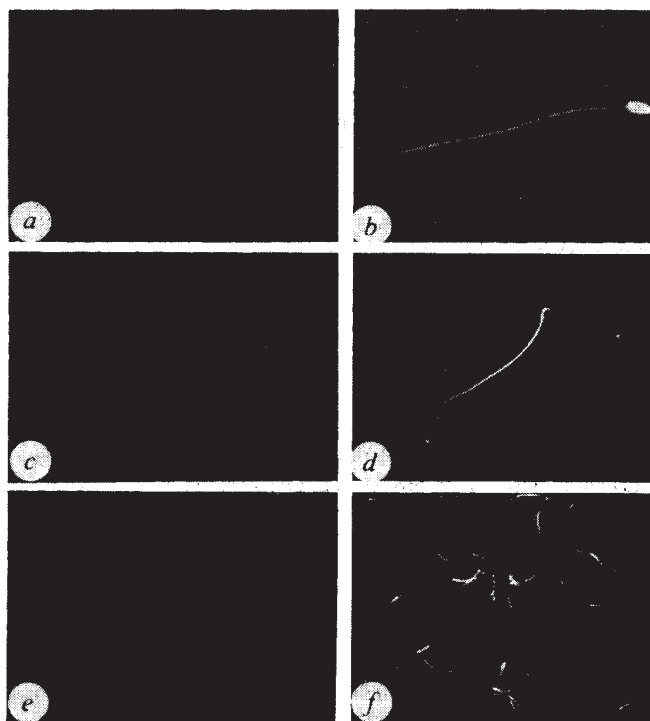


Fig. 2 Reactivity of SSA with sperm of three species visualized by IIF *a* and *b*, sea urchin; *c* and *d*, rat; *e* and *f*, turkey. *a*, *c* and *e*, preimmune serum; *b*, *d* and *f*, SSA. Sperm were fixed and washed as in Fig. 1 legend, then incubated for 1 h in preimmune serum (1/100) or SSA (1/100), washed three times in 1% normal rabbit serum in PBS, incubated 15 min in 1:10 fluorescent goat anti-rabbit antiserum (Antibodies Incorporated), and washed three times in PBS over a 30-min period. The cells were then resuspended in PBS and observed and photographed on GAF 500 film with a Zeiss fluorescent microscope equipped with an Osram mercury lamp. The fluorescence appears over the entire cell surface. Pre-immune serum did not react.

unfixed sperm without adjuvant, and this antiserum, after absorption, had the same specific reactivity as the SSA.

The possibility existed that the supposedly sperm-specific activity was not due to the recognition of a surface antigen but resulted from the cross-reactivity of male accessory gland secretions. However, no reactivity was detected when sea urchin seminal fluid was diffused in agar against the SSA. Another possibility was that the SSA was recognizing H-Y antigen, the male sex-determining antigen present on the sperm (and all other cells) of all species in which the male is the heterogametic sex^{3,4}. To answer this question the following experiments were performed. First, the serum was extensively absorbed using sea urchin male non-gametic tissues (coelomocytes and tube feet); the activity of the SSA was not affected. Second, the antiserum

was extensively absorbed using splenocytes from two male rats (the standard procedure for absorbing H-Y antibodies⁵); there was no loss of activity of the SSA. It has been reported that H-Y activity is limited in distribution to the region of the acrosomal cap in mammalian sperm⁶. We found, however, that SSA activity was distributed over the entire surface of rat and hamster sperm, as judged by immunoperoxidase localization using phase-contrast microscopy at $\times 1,000$ magnification and by IIF. Finally, the serum reacted with turkey sperm by both the immunoperoxidase and IIF (Fig. 2*e*, *f*) procedures, proving conclusively that the SSA is not recognizing H-Y activity, because in birds the heterogametic sex, and hence the bearer of H-Y (H-W in birds), is the female^{3,4}.

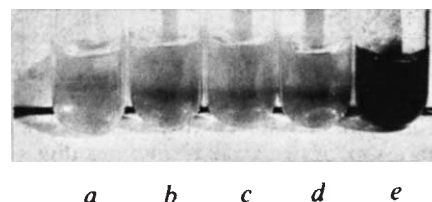


Fig. 3 No reaction product was deposited when the immunoperoxidase procedure was performed using sea urchin sperm in the absence of one of the following reactants: *a*, hydrogen peroxide; *b*, SSA; *c*, horseradish peroxidase. *d*, Preimmune serum, and *e*, SSA, all reagents included.

In mammals, cross-reacting antigens exist among nervous tissue, sperm and preimplantation embryos⁷. However, we found that the SSA did not react with fixed or unfixed rat brain. Some sperm, such as those of the rabbit, nonspecifically bind the Fc region of antibodies⁸. To exclude this as a possibility for the activity of our SSA, we tested two other antisera raised against two different genus-specific sea urchin sperm surface glycoproteins (*S. purpuratus*⁹). Sperm of 16 species, representing four phyla, did not react with these other antisera, showing that the activity of the SSA did not result from nonspecific binding of the Fc region of the rabbit immunoglobulins. To investigate the possibility that the SSA was reacting with cell-surface tubulin, we reacted it with *Chlamydomonas reinhardtii* cells and mussel gill tissue (*Mytilus californicus*), two cell types reported to have tubulin on their outer surface¹⁰⁻¹³. Both cell types gave a negative reaction by the immunoperoxidase assay. The results here suggest that common cross-reactive antigenic determinant(s) exist which are specific to the surface of animal sperm.

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Table 2 Cells and tissues which did not react with SSA as assessed by immunoperoxidase

1. Cell lines
DS8 = Mouse mammary tumour
A549 = Human lung tumour
ATC103 = Iguana heart
B104 = Rat neuroblastoma
DC2 = <i>Drosophila</i> Schneider cells
2. <i>Chlamydomonas reinhardtii</i> cells
3. Mussel gill tissue (<i>Mytilus californicus</i>)
4. Sliced rat brain tissue
5. Dissociated blastomeres of sea urchin hatched blastula (<i>S. purpuratus</i>)

See Fig. 1 legend for methods.

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Retroviral antigens on gs^- chf^- leukocytes

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It has recently been suggested that the endogenous retroviruses present in many different species might be involved during stimulation of the immune system of their hosts¹⁻³. We have now studied the expression of two avian retroviral antigens p27 and gp85 in chicken lymphoid cells by indirect immunofluorescence (IIF) and by complement-dependent microcytotoxicity (CDM). We have now found that these viral antigens are expressed in peripheral blood leukocytes of adults and embryos and in splenic and bursal lymphocytes of Spafas gs^- chf^- chickens but they are not expressed in fibroblasts cultured from the feather follicles of the same individual adult birds nor in fibroblasts cultured from embryos of the same flock. The differential expression of viral antigens in leukocytes may be related to a specific property or function of these cells.

We wished to study the possible modifications of the expression of avian retroviral antigens by chicken lymphoid cells during the immune response, but, using outbred gs^+ chf^+ birds, we observed that their lymphoid tissues frequently expressed considerable quantities of p27 and gp85 as estimated by IIF (see Table 1). Splenic cells from some 20 birds of ages ranging from 3 weeks to 6 months were 30–100% positive for either antigen; bursal cells from the same birds were more variable, some samples being negative for one or both of the antigens, others consisting of 100% positive cells. Only a few samples of peripheral blood leukocytes from these birds were examined but they were uniformly strongly positive ($\geq 80\%$) for both antigens. These results agree with those of Chen and Hanafusa⁴ who used radioimmunoassay techniques to show that p27 was expressed in greater amounts by the lymphocytes of such chickens than by any other tissue. We therefore decided to use Spafas chickens which, although they contain the endogenous viral genome, do not usually express the corresponding antigens⁵⁻⁷.

In an initial experiment, leukocytes from peripheral blood of adult Spafas chickens were examined by IIF for expression of viral antigens (see Table 1). A considerable proportion of peripheral blood leukocytes from all four of the birds reacted with the anti-gp85 serum (50–90%) and with the anti-p27 serum (40–70%) and these percentages remained fairly stable for the individual birds over the experimental period. The birds were killed and smears prepared from mechanically disrupted splenic and bursal tissue and compared with the peripheral blood samples. The percentage of positive cells was never higher in these tissues than in the corresponding blood.

These results were confirmed using a CDM assay as described by Mohanakumar *et al.*⁸. A series of Spafas chickens were the source of peripheral blood leukocytes and these were examined by the CDM test using the same antisera as used in the IIF.

In these conditions, the anti-p27 serum caused a specific cytotoxicity of 50–80% of Spafas peripheral blood leukocytes at a dilution of 1:20 and had an end point titre of 80–160. The anti-gp85 serum showed greater reactivity, regularly killing some 90% of the target cells at dilutions up to 1:5,000 and having an end point titre of 20,000–40,000 (Fig. 1). The specificity of this reaction was verified by absorption of the serum against cells with known expression of viral antigens. This

activity could be absorbed by Spafas blood leukocytes or by mammalian cells transformed by Rous sarcoma virus and expressing viral antigens but not by analogous untransformed cells nor by Spafas fibroblasts even after 5-bromodeoxyuridine (BUdR) treatment. Similar results, although at lower titre, were obtained with antisera raised in rabbits using a different preparation of p27 and gp85 antigens.

As a further control, the reactivity of the sera towards Spafas embryo fibroblasts was tested by IIF. Neither p27 nor gp85 was ever expressed over 12 culture passages, even after culturing the cells for 6 days in the presence of BUdR ($4 \mu\text{g ml}^{-1}$). We also cultivated fibroblasts from the feather follicles⁹ of individual Spafas chickens which provided the blood leukocytes for comparisons over the culture period. These fibroblasts also showed no reactivity by IIF for either antigen even after BUdR treatment and were incapable of absorbing the reactivity of the sera for leukocytes of the same individual chicken (Fig. 1).

The expression of these antigens by peripheral blood leukocytes has regularly been observed at all ages from newly hatched chicks to birds of over 1 year old. We have also tested some samples from chick embryos, and have found by IIF that white blood cells are already positive for both antigens in 10-day-old embryos, the earliest date at which we could obtain adequate samples. An additional technique has been used to detect these viral antigens in Spafas lymphocytes. This consisted of competitive radioimmune assays using 'purified' labelled antigens and competition with 'cold' antigens from cells. For p27, lymphocytes showed weak activity and muscle, heart, liver, kidney and lung were negative. For gp85, lymphocytes showed greater activity than transformed mammalian cells used as positive controls, other tissues being negative.

The difference in expression of retroviral antigens between Spafas fibroblasts and lymphocytes, although both cell types contain the endogenous viral genome, indicates that some control over this expression must be exerted at the transcriptional or post-transcriptional level. Our results suggest that this regulation acts differently in different tissues of the same bird, as fibroblasts from a given individual can be totally negative whereas the leukocytes from the same bird express considerable quantities of both p27 and gp85. A similar observation has been reported for BALB/Mo mice infected at an early embryonic stage with Moloney leukaemia virus. After birth, viral antigen was found to be strongly expressed by splenic and thymic cells, but not by those of other tissues tested (liver, kidney and brain), although the viral genome was present in the DNA of all these organs¹⁰. This system is, however, not a natural

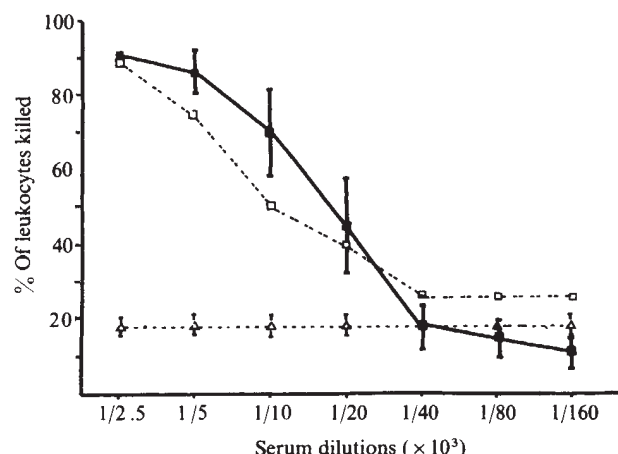


Fig. 1 Complement-dependent microcytotoxicity assay of anti-gp85 (AMV) serum against Spafas peripheral blood leukocytes. ■, Unabsorbed serum ($n = 12$); □, serum absorbed on feather follicle fibroblasts from the same chicken (duplicate measures); △, serum absorbed on Spafas leukocytes ($n = 4$). Bars represent s.d. In the absence of complement or with control negative sera the percentage of leukocytes killed is always $\leq 20\%$.

Table 1 Expression of endogenous viral antigens in gs^- chf^- chicken lymphocytes

Chicken	Age	Tissue	Antiserum	Test	Reactivity
Outbred gs^+ chf^+	3 weeks–6 months	Spleen	Anti-p27	IF	+30–100%
		bursa	and		+10–100%
Spafas gs^- chf^-	4 months	Spleen bursa blood	Anti-gp85	IF	+≥80%
			Anti-p27		+40–70%
			Anti-gp85	CDM	+50–90%
			Anti-p27		+50–80%
Spafas gs^- chf^-	0–1 yr	Blood	Anti-gp85	IF	+90%
			Anti-p27		+
			Anti-gp85	IF	+
			Anti-p27		+
Spafas gs^- chf^-	10–18-day-old embryo	Blood	Anti-gp85	IF	–
			Anti-p27		–
			Anti-gp85	IF	–
			Anti-p27		–
Spafas gs^- chf^-	Embryo	Fibroblast cultures 12 passages $\pm$ BUdR	Anti-p27	IF	–
			and		–
			Anti-gp85	IF	–
			Anti-gp85		–
Spafas gs^- chf^-	Adult	Feather follicle fibroblast cultures $\pm$ BUdR	Anti-p27	IF	–
			and		–
			Anti-gp85	IF	–
			Anti-gp85		–

For IIF testing, blood samples were taken from four adult (4-month-old) Spafas chickens at 2–3-day intervals over a 15-day period. The blood was taken from the wing vein and collected into citrate. The leukocytes were separated either by allowing the cells to settle at 37 °C and recovery of the buffy coat, or by centrifugation through Ficoll-paque (Pharmacia) at 1,500 r.p.m. for 15 min. Identical results were obtained using either method. After washing with phosphate-buffered saline (PBS), pH 7.4, the cells were smeared on to microscope slides, air dried, fixed in buffered formaldehyde (3.7 % w/v) for 3 min, rinsed and postfixed in dry acetone for 10 min. Dilutions of antisera to p27 or gp85 raised in rabbits to purified polypeptides from avian myeloblastosis virus or control sera were then allowed to react with the fixed smears for 45 min at 37 °C. After washing with PBS, the reaction was visualized by a further incubation with fluorescein-labelled goat anti-rabbit immunoglobulin serum (Behring, dilution 1:50) for 30 min at room temperature. The washed specimens were then examined under a Zeiss Ultraphot II fluorescence microscope. In some cases the surface expression of the antigens was estimated by the use of unfixed cell suspensions in a similar protocol. Control samples of cultured cell lines with known expression of the viral antigens were always included in each series. For cytotoxicity testing, the separated leukocytes were resuspended at 6×10^6 cells ml^{-1} in RPMI 1640 medium supplemented with 10% inactivated fetal calf serum. This suspension (1 μ l) was introduced under liquid paraffin into each well of a 60-well Falcon microtitre plate together with 1 μ l of diluted antiserum. After 30 min at room temperature, 5 μ l of rabbit complement (Gibco), previously absorbed on adult chicken erythrocytes and brought to a dilution of 1:3, was added to each well and the incubation continued for 45 min. After this the dead cells were stained by the introduction of 5 μ l of a solution of eosin to each well, then, 2 min later, the cells were fixed by the introduction of 2 μ l of buffered formaldehyde (30% w/v, pH 7.0). The reactions were read under the phase-contrast microscope.

transmission of a strictly endogenous virus. In the same system there was no stimulation of viral expression in actively proliferating hepatocytes¹¹, arguing against the hypothesis that endogenous virus expression might be simply associated with cellular proliferation.

The association of the expression of endogenous viral antigens with cells intimately involved in the animal's immune responses suggests some relationship between these molecules and the cell-surface (glyco)proteins implicated in immune reactions. The fact that viral antigens are expressed on most leukocytes suggests that these molecules may be involved in a general function of these cells rather than affecting only those cells and their clones specifically sensitized by antigen. Recently, Astrin *et al.*¹² have successfully reared to breeding age a healthy cockerel that has no endogenous viral DNA in its genome. It would be of interest to test the immune response of such birds and to compare it with that of birds carrying the endogenous virus.

Possibly the observed effect can be explained on the basis of artefact due to the culturing procedure itself. Fibroblasts might be negative for virus expression because of trypsinization or culturing. This explanation is, however, improbable as gs^+ chf^+ fibroblasts in these same conditions retain viral expression suggesting that gs^- chf^- fibroblasts are inherently negative. A more likely reason for the differential viral antigen expression may involve one of several control levels. For example, viral

RNA may be constitutively expressed in lymphocytes whereas it may require induction in fibroblasts.

Our results suggest that p27 expression takes place on the surface of lymphocytes. Although the 'usual' mode of expression of p27 is intracellular, a large precursor polypeptide containing the sequence of p27 has been shown to be present on the surface of cells infected by RSV, although in this case p27 antigenicity could not be demonstrated¹³. Alternatively, the lymphocyte plasma membrane might permit the insertion of p27 although that of fibroblasts does not.

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Genes which control cell proliferation in the yeast *Saccharomyces cerevisiae*

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In many eukaryotes it is thought that cell proliferation is regulated at a point in G_1 close to the initiation of DNA synthesis¹. Hartwell^{2,3} and his colleagues have shown such a point in G_1 phase in the budding yeast, *Saccharomyces cerevisiae*, defined by the *cdc 28* mutation. He has termed this point 'start' and showed that for cells to proceed beyond start, initiate DNA synthesis and produce a bud, various conditions must be met. Two of these conditions are the presence of adequate nutrients in the medium and the attainment of a critical size. We identify here some of the genes controlling start by isolating mutants which are altered with respect to the conditions in which start occurs. Two types of mutant have been isolated. One results in bud initiation when the parent cell is only half the size at which bud initiation occurs in wild-type cells. Such mutants define a single gene, *whi-1*, and they are apparently analogous to the size mutants isolated by Nurse and his colleagues^{4–6} in *Schizosaccharomyces pombe*. A second type of mutation affects a second gene, *whi-2*, which is involved in the mechanism whereby cells arrest in G_1 in stationary phase. *whi-2* cells growing exponentially initiate buds at the same size as wild-type cells. In stationary phase, however, *whi-2* cells, unlike wild-type cells, are predominantly budded and are smaller than wild-type cells.

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Small cell-size mutants of strain X2180-1A were isolated by a procedure involving cell size separation by zonal centrifugation together with the use of the mating hormone α factor. This procedure is described in detail elsewhere⁷. Three mutants, designated *whi A1*, *whi C7* and *whi D3*, which were recovered from different mutagenized cultures and are therefore independent mutations, were examined in detail. Parent cell volume of these mutants is shown in Table 1, together with dry mass measurements obtained by scanning interferometry and cell protein content for the wild type and the mutant *whi-A1*. Mutant cell size, whether measured by volume, protein content or dry mass, is approximately one-half that of the wild type. Volume was routinely measured as a parameter of cell size in further determinations.

Table 1 Cell sizes of wild type and *whi-1*⁻ mutants in exponential growth phase

Strain	Mother cell volume* (μm^3)	Total cell protein† ($\text{g} \times 10^{13}$)	Dry mass‡ at bud initiation (arbitrary units)
X2180-1A	47 ± 2	7.5	11.45 ± 0.38
<i>whi-A1</i>	25 ± 1	4.7	5.39 ± 0.48
<i>whi C7</i>	26 ± 2	—	—
<i>whi D3</i>	20 ± 1	—	—

* Cells were inoculated from a YEPD plate into 2.5 ml YEPD medium in a 50 ml Erlenmeyer flask. The culture was shaken overnight at 25 °C. The next day 0.1 ml of the late log phase culture was re-inoculated into 2.5 ml fresh medium and cultured in the same conditions for a further 5 h. Photomicrographs were prepared with a phase-contrast microscope at +400 magnification. Cells were concentrated by centrifugation if necessary. The negatives were projected and the major (a) and minor (b) axis of budded mother cells measured. The final magnification was measured using a photomicrograph of a stage graticule. Volume was calculated assuming a prolate spheroid shape according to the formula $4/3\pi ab^2$. Sufficient cells were measured so that the s.e.m. shown, was below 5% of the mean. This involved a minimum of 25 cells.

† Cells were cultured as above. Cell number per ml was measured with a Coulter counter following 10-s sonication to separate clumped cells. To measure protein per ml the cells were washed twice in distilled water and dissolved in 1 M NaOH at 100 °C. Protein was estimated using the Folin test¹⁴.

‡ Unbudded stationary phase cells were diluted into fresh YEPD medium. At intervals samples were removed and mounted in a thin layer of gelatin on a slide¹⁵. Using a scanning interferometer¹⁶, the dry mass of cells which had just produced buds was measured. Measurements were continued until over 95% of the cells were budded. The errors are s.e.m.

Each mutant was crossed with XJB17-1 (*arg 9*, *his 4*, *ura 1*, *ilv 3*) and AN 33 (*arg thr*) both of which are α mating type and produce buds at the same parent cell volume as X2180-1A. The parent cell volume for wild-type diploid cells is 81 μm^3 (Table 2). (A similar pattern of results is obtained if only zero bud scar class cells are considered.) The heterozygous diploids had cell volumes intermediate between those of wild-type haploid cells and diploid cells (Table 2). Thus the three mutant alleles are semi-dominant to the wild-type allele.

The heterozygous diploids were sporulated and tetrads dissected. The small phenotype segregated 2:2 in all tetrads showing that in each mutant it was due to a single nuclear mutation. Small progeny with appropriate combinations of mating type and auxotrophic markers were crossed together and diploids selected by prototrophic selection. These diploids all had a parent cell volume similar to that of wild-type haploids (Table 2).

Heteroallelic mutant diploids were sporulated and cell volume at bud initiation measured in the individual spore clones of the resulting tetrads. At least 20 tetrads were analysed in each of the three combinations but no wild-type recombinant pro-

geny were observed. Thus, the mutations probably affect the same gene, *whi-1*, although two closely linked genes could be involved.

If more than one gene is involved sequentially in the synthesis of a product controlling cell size, then the data shown in the last three lines of Table 2 may be used for complementation tests. Although the alleles are apparently showing dosage effects, if two alleles are in the same cistron then the heteroallelic diploid should be the same size as the homozygous diploid. If the alleles are in different cistrons then the heteroallelic diploid should be intermediate in size between the homozygous and heterozygous diploid. The data show the heteroallelic to be the same size as the homozygous diploid, showing the alleles to be in the same cistron. However, the predicted difference is small and may not be resolvable in the tests.

An alternative interpretation of the data, precluding the use of complementation tests, arises from the observation that the homozygous diploid is still twice the size of the mutant haploid even though neither will be producing any normal product from the *whi-1* gene. There is clearly an element of size control remaining which could be explained if there was a second gene so far undefined by mutation. The product of a second gene would act in parallel to that of the *whi-1* gene so that each would affect cell size directly. The size of the cell at bud initiation would therefore be proportional to the combined number of these two genes. The wild-type haploid would thus contain two genes and the wild-type diploid, four. The *whi-1*⁻ haploid retains one gene, the homozygous diploid two and the heterozygous diploid three. The observed sizes of 20–25 μm^3 (*whi-1*⁻ haploid), 45–50 μm^3 (wild-type haploid and homozygous mutant diploid), 60–65 μm^3 (heterozygous diploid) and 81 μm^3 (wild-type diploid) agree well with this interpretation. The results also predict that a second class of mutant, if isolated, could only be distinguished from *whi-1* mutants by recombination. Complementation could not be used, because the heteroallelic diploid would contain two functional genes whether or not the alleles were in the same or different genes.

The mutation *whi-2* apparently occurred spontaneously in strain M11. Whereas *whi-1* cells are smaller than wild type in both exponential growth and stationary phases, *whi-2*⁻ mutants are approximately one-half the size of wild-type cells only in stationary phase (Table 3). In stationary phase these cells also differ from wild-type cells as: (1) the budding index is 24% compared with 1.6% of wild-type cells (Table 3). (2) The final cell density is approximately twice that of wild-type cells. (3) The cells have the phase-dark appearance characteristic of exponentially growing cells rather than the normal phase-bright appearance of stationary-phase cells (Fig. 1).

This phenotype suggests a lesion causing defective G₁ arrest. The cells undergo at least one more cycle of division in the absence of growth and consequently become smaller than wild-type cells.

whi-2⁻ cells were crossed with 4/4D, a wild-type strain. In stationary phase the resulting diploid was phase-bright,

Table 2 Mother cell volumes of *whi-1*⁻ heterozygous and homozygous diploids growing in exponential phase

Strain	Constitution	Volume
XJB17.1	<i>whi-1</i> ⁺	50 ± 3
C276	<i>whi-1</i> ⁺ / <i>whi-1</i> ⁺	81 ± 3
<i>whi A1</i> × XJB17.1	<i>whi-A1</i> ⁻ / <i>whi-1</i> ⁺	60 ± 2
<i>whi C7</i> × XJB17.1	<i>whi C7</i> ⁻ / <i>whi-1</i> ⁺	64 ± 2
<i>whi D3</i> × XJB17.1	<i>whi D3</i> ⁻ / <i>whi-1</i> ⁺	65 ± 3
<i>whi A1.1a</i> × <i>whi A1.1b</i>	<i>whi A1</i> ⁻ / <i>whi A1</i> ⁻	45 ± 2
<i>whi C7.7d</i> × <i>whi C7.8d</i>	<i>whi C7</i> ⁻ / <i>whi C7</i> ⁻	50 ± 2
<i>whi D3.4b</i> × <i>whi D3.9c</i>	<i>whi D3</i> ⁻ / <i>whi D3</i> ⁻	47 ± 2
<i>whi A1.1b</i> × <i>whi C7.6c</i>	<i>whi A1</i> ⁻ / <i>whi C7</i> ⁻	51 ± 2
<i>whi C7.5b</i> × <i>whi D3-22</i>	<i>whi C7</i> ⁻ / <i>whi D3</i> ⁻	44 ± 2
<i>whi A1.5d</i> × <i>whi D3-22</i>	<i>whi A1</i> ⁻ / <i>whi D3</i> ⁻	46 ± 3

Culture conditions are described in Table 1 legend.

Table 3 Cell size, final number per ml, percentage budding and percentage viable cells in stationary-phase cultures

Strain	Constitution	Cell volume (μm^3)	Final cell no. $\text{ml}^{-1}(\times 10^9)$	% Budding	% Viable cells
X2180-1A	<i>whi 1⁺ whi 2⁺</i>	36 ± 3	1.1	2	55
C276	<i>whi 1⁺ whi 2⁺ / whi 1⁺ whi 2⁺</i>	68 ± 2	0.49	10	65
M11	<i>whi 1⁺ whi 2⁻</i>	24 ± 1	1.97	24	30
S673a	<i>whi 1⁻ whi 2⁺</i>	15 ± 1	1.98	13	21
XJB.9d.4a	<i>whi 1⁻ whi 2⁻</i>	8 ± 1	3.60	31	10
XJB.9d.4a $\times$ M11.13d.5d	<i>whi 1⁻ whi 2⁻ / whi 1⁻ whi 2⁻</i>	19 ± 1	1.95	47	5

Cells were inoculated from a YEPD plate into 2.5 ml YEPD medium in a 50-ml Erlenmeyer flask. They were cultured for 48 h at 25 °C, by which time cell number had stopped increasing and the culture considered to be in stationary phase. Cell volume of all cells was measured as described in Table 1 legend. After 10 s sonication, cell number was determined using a Coulter counter, percentage budding by microscopic examination, and per cent viable cells by plating out on YEPD plates at a suitable dilution and counting colonies that had appeared after 3 days.

unbudded and the mean cell volume was $62 \pm 4 \mu\text{m}^3$, only slightly less than the wild-type diploid in stationary phase ($68 \mu\text{m}^3$, Table 3). The mutation is therefore recessive. The diploid was sporulated and tetrads dissected; the phenotype segregated 2:2 in all 30 tetrads examined and therefore results from a single nuclear mutation.

M11 is a strain derived from DR19/T7 which⁷ lacks the 2- μm plasmid⁸. This trait is not associated with its absence. First, the trait segregates as a nuclear gene whereas the 2- μm plasmid is inherited cytoplasmically. Second, *whi-2⁻* segregants from the above cross were shown to have received the 2- μm plasmid when they were screened for its presence by buoyant density centrifugation.

A strain carrying the *whi-2* mutation was crossed with one carrying the *whi-1* mutation, the resulting diploid was sporulated and spore clones were grown to stationary phase and analysed for parent cell size. Wild-type recombinants were observed in some tetrads, which also contained spore clones

smaller in stationary phase than either *whi-1⁻* or *whi-2⁻* cells (Table 3). These are the reciprocal recombinant *whi-1⁻ whi-2⁻*. The numbers of complete tetrad types was 1 Pd: 3 NPDs: 13 Ts indicating unlinked genes. That the putative *whi-1⁻ whi-2⁻* was a double mutant was confirmed in a further cross with wild type where the expected single mutant recombinants were recovered.

The stationary-phase appearance of wild-type, *whi-1⁻*, *whi-2⁻* and *whi-1⁻ whi-2⁻* cells is shown in Fig. 1. *whi-1⁻ whi-2⁻* mutants are much smaller than wild-type cells (Table 3). The high budding index (31%, Table 3), high final cell density (Table 3) and phase-dark appearance suggest continuing division. Cell size is only $8 \mu\text{m}^3$ (Table 3) compared with the $38\text{-}\mu\text{m}^3$ cell volume of wild-type cells in stationary phase and $50 \mu\text{m}^3$ at which wild-type cells produce buds. Clearly, *whi-1⁻ whi-2⁻* cells have lost much of their control over cell division.

The sizes of strains containing the *whi-2⁻* allele growing in exponential phase are shown in Table 4. The sizes are the same as the corresponding *whi-2⁺* strain indicating an abnormal

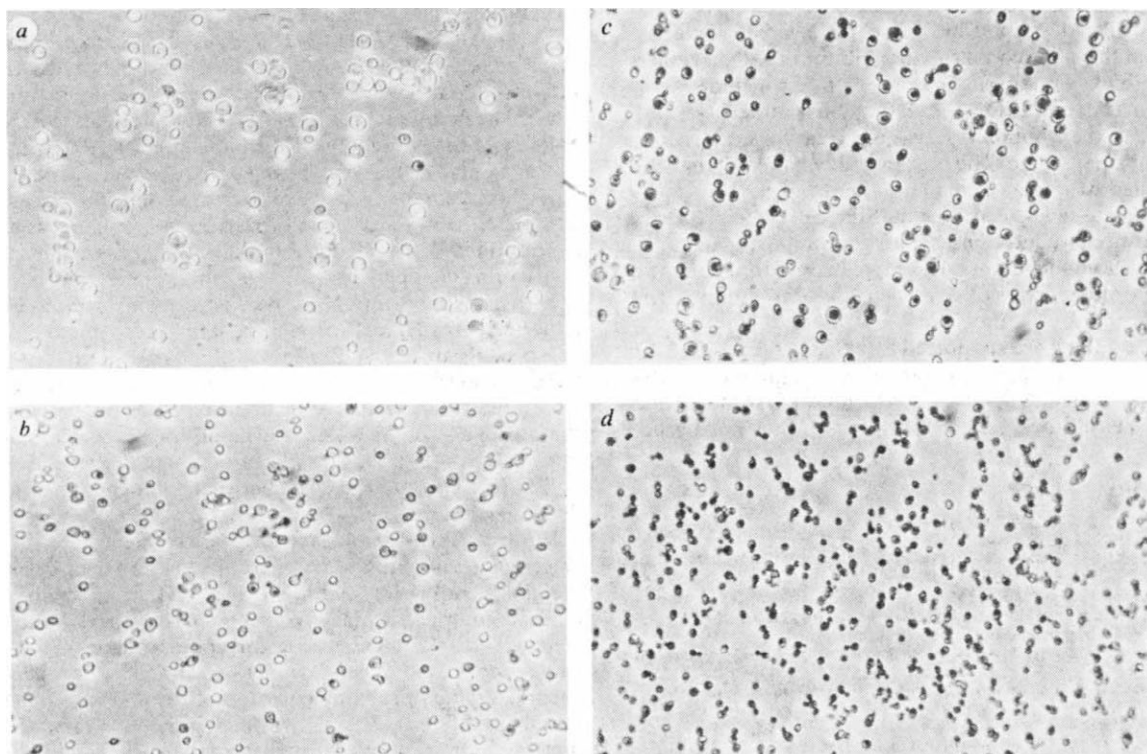


Fig. 1 Phase-contrast pictures of stationary-phase haploid cells. *a*, Wild-type cells; *b*, *whi-1⁻*; *c*, *whi-2⁻*; *d*, *whi-1⁻ whi-2⁻*. Stationary-phase cultures were prepared as described in Table 3 legend and photographed with a phase-contrast microscope. Final magnification $\times 420$.

phenotype is only expressed in stationary phase. This suggests that *whi-2* is not the gene whose existence was inferred from the size of *whi-1* strains. However, to investigate this further, the diploid *whi-1⁻ whi-2⁻/whi-1⁻ whi-2⁻* was isolated by mating double mutant haploids carrying complementary auxotrophic requirements. In stationary phase this diploid was extremely small (19 μm^3), only 52% of the size of the wild-type haploid in similar condition. The doubly homozygous mutant diploid had the additional characteristic of the double mutant haploid—a high budding index. Furthermore, the size increased to that of a *whi-1⁻/whi-1⁻* strain when inoculated into fresh medium. In stationary phase the *whi-1⁻ whi-2⁻/whi-1⁻ whi-2⁻* homozygous diploid is larger than the *whi-1⁻ whi-2⁻* haploid. Therefore, either an element remains which ensures an increase in cell size in the diploid, or no active size regulation remains and size increase is a result of a physical requirement for the accommodation of cellular organelles.

Table 4 Cell volumes of *whi-2⁻* strains growing in exponential phase

Strain	Constitution	Cell size
M11	<i>whi-2⁻</i>	45 $\pm$ 2
M11/44D	<i>whi-2⁻/whi-2⁺</i>	90 $\pm$ 4
XJB.9d.4a	<i>whi-1⁻ whi-2⁻</i>	18 $\pm$ 1
XJB.9d.4a/	<i>whi-1⁻ whi-2⁻</i>	40 $\pm$ 2
XJB.9d.10a	<i>whi-1⁻ whi-2⁻</i>	

Cells were grown in exponential phase as described in Fig. 1 legend.

The loss of size control and/or G_1 arrest would reduce cell viability. Thus, wild-type cells are more viable than either *whi-1⁻* or *whi-2⁻* cells, which in turn are more viable than the *whi-1⁻ whi-2⁻* double mutants (Table 3). However, many cells remain viable, possibly due to the action of the, as yet, undefined gene which may act in a similar manner to *whi-1*.

These studies show that cell size and G_1 arrest are under separate genetic control. One gene, *whi-1*, controls cell size and its action is dose-dependent, as might be expected if cell size is normally proportional to ploidy⁹⁻¹³. This gene may correspond to the *wee-1* gene described by Nurse⁶ in *S. pombe* and the conclusion that this gene codes for an inhibitor of cell division is consistent with our findings. Fantes *et al.*⁹ concluded that elements must exist on the DNA exerting their influence on cell size according to their number. It was not clear how many such elements exist to a genome. The present study indicates that cell size is controlled by one or a small number of genes.

The second gene, *whi-2*, ensures that the cell cycle arrests in G_1 when nutritional conditions no longer support growth. A *whi-1⁻ whi-2⁻* double mutant is defective in both functions and has lost much of the control over cell division exhibited by wild-type cells.

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Conservation and rearrangement of mitochondrial structural gene sequences

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Mitochondria contain the simplest DNA molecules that are present in eukaryotes. Mitochondrial DNA (mtDNA) is easily purified, and is an important model system for studying eukaryote gene structure and basic molecular processes. The protein sequences of mitochondrial gene products have been shown to be conserved from yeast¹⁻³ to man⁴, and there are definite similarities at the DNA sequence level. In contrast, the overall organization of the mitochondrial genome is drastically different in these organisms. To understand this, we need to extend work on mtDNA to a wider range of species. We have chosen to study the mtDNA of *Aspergillus nidulans* because a particularly comprehensive analysis of this system can be achieved using genetics as well as biochemistry, and like most eukaryotes it is an obligate aerobe, whereas *Saccharomyces cerevisiae* is not. We have investigated whether defined pieces of particular yeast mitochondrial genes show enough homology to *Aspergillus* mtDNA fragments to enable the corresponding *Aspergillus* genes to be located on the physical map. The results reported here show that this is the case for all five genes tested, and present the first data on the physical organization of the structural genes in the mitochondrial genome of *A. nidulans*.

The yeast DNAs that we have used to locate homologous *Aspergillus* sequences by hybridization are derived from the mtDNA of particular well defined *petite* (p^-) deletion mutants of yeast. The deletion is usually large and sometimes leaves as little as 0.1% of the mitochondrial genome intact, so that single genes or fragments of genes may be the only sequences remaining. The molecules actually used as probes for homology were 5'-end-labelled single strands of defined restriction fragments prepared as described in Fig. 1 legend. The five *petite* strains and the restriction fragments used are shown in Fig. 2.

A. nidulans mtDNA was cut with the restriction endonucleases *Hind*III or *Hae*III, and the fragments separated in an agarose gel and transferred to diazobenzyloxymethyl (DBM) paper^{5,6}. After hybridization and autoradiography, we were able to show that specific yeast gene probes hybridized preferentially to particular restriction fragments from *A. nidulans* mtDNA (Fig. 1). This indicates a high degree of homology at the DNA sequence level between mitochondrial genes of these species.

We used this homology with yeast sequences to locate five *Aspergillus* mitochondrial structural genes on the physical map; the genes coding for subunits I, II and III of the cytochrome *c* oxidase complex, which we name [*oxiA*], [*oxiB*] and [*oxiC*], respectively, the gene coding for apocytochrome *b*, which we name [*cobA*], and a gene coding for subunit VI of the ATPase complex, which probably corresponds to the gene [*oliA*], previously identified genetically as giving rise to oligomycin-resistant mutants⁷. The parts of the yeast genes to which the five probes correspond are shown in Fig. 2. A restriction map of *Aspergillus* mtDNA is given in Fig. 3.

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Fig. 1 Autoradiographs of Southern transfers of restriction endonuclease digests of *A. nidulans* mtDNA after hybridization to yeast mtDNA fragments. Mitochondrial DNA of *A. nidulans* strain *bi A-1* was prepared by a modification of the method of Cummings *et al.*¹⁹. After restriction endonuclease digestion in 6.6 mM Tris-HCl pH 7.4, 6.6 mM MgCl₂, 50 mM NaCl and 1 mM dithiothreitol, 2 µg of DNA was loaded per track of an 0.8% agarose gel²⁰. After electrophoresis the gel was stained in ethidium bromide and photographed, then transferred to DBM paper⁶ by the method of Southern⁵. Yeast mitochondria were prepared as described by Faye *et al.*²¹, and mitochondrial DNA purified as in Sanders *et al.*²². Restriction enzyme digests (all enzymes from Bethesda Research Laboratories) of yeast mtDNA were 5' end-labelled with [³²P]ATP (NEN)²³ using polynucleotide kinase (Bethesda Research Laboratories). The labelled fragments were denatured in 90% formamide and run into a 6% polyacrylamide gel to separate the strands of each fragment. The area of gel containing the strand to be used as a hybridization probe was cut out after identification by autoradiography and the DNA eluted from the gel and ethanol precipitated²³. Preparation of the DBM paper and hybridization of labelled yeast mtDNA fragment single strands to the Southern transfer were carried out following Wahl *et al.*⁶, using 10% dextran sulphate in the hybridization mixture to improve the efficiency of hybridization⁶. The fragments used as probes for the various tracks, identified by their gene of origin, are *a*₁, *a*₂ and *e*, *oxi*1; *b* and *f*, *oxi*2; *c* and *g*, *cob*; *d* and *h*, *oli*2; *i* and *j*, *oxi*3. *a*₁ and *a*₂ were from different series of experiments using formamide from Fluka and Merck, respectively. Tracks *a*₁–*d* and *i* are of Southern transfers of *Hind*III digests, tracks *e*–*h* and *j* of Southern transfers of *Hae*III digests. The positions where DNA bands were present in the ethidium bromide-stained agarose gels before the Southern transfers are marked and numbered.

The yeast genes for cytochrome *c* oxidase subunits I, II and III are *oxi*3, *oxi*1 and *oxi*2, respectively. The probes for both *oxi*3 and *oxi*1 can be seen in Fig. 1 to hybridize to the large *Aspergillus* *Hind*III fragment 1 and to the 3,500-base pair *Hae*III fragment 3. This places the promoter-proximal half of [*oxi*B] (= *oxi*1) and the promoter-distal half of [*oxi*A] (= *oxi*3) within this *Hae*III fragment. The *oxi*2 probe hybridizes with *Aspergillus* mtDNA *Hind*III fragment 3 and *Hae*III fragment 4, placing the [*oxi*C] gene in the 2,500-base pair region between the large and small mitochondrial ribosome RNAs, together with a cluster of tRNA genes⁸.

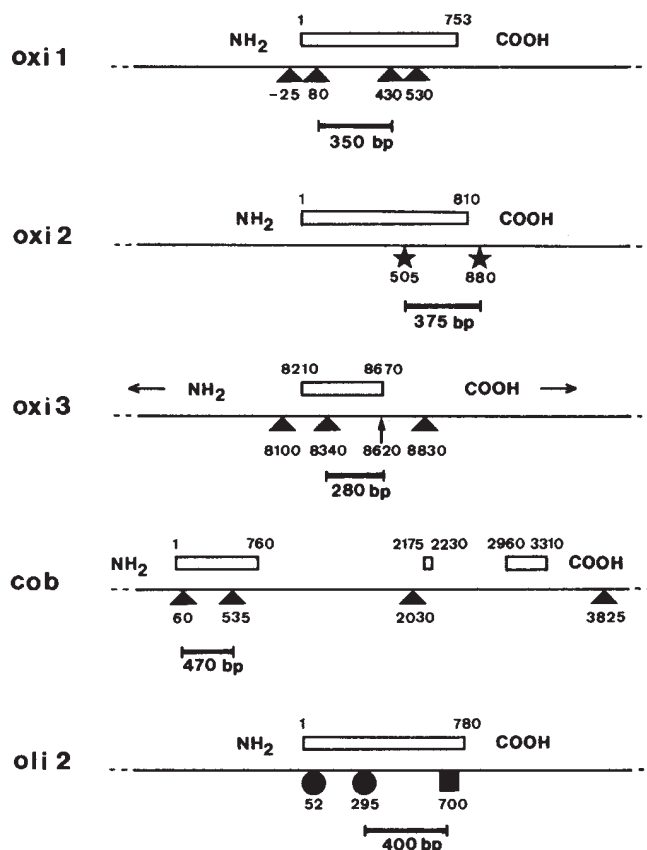
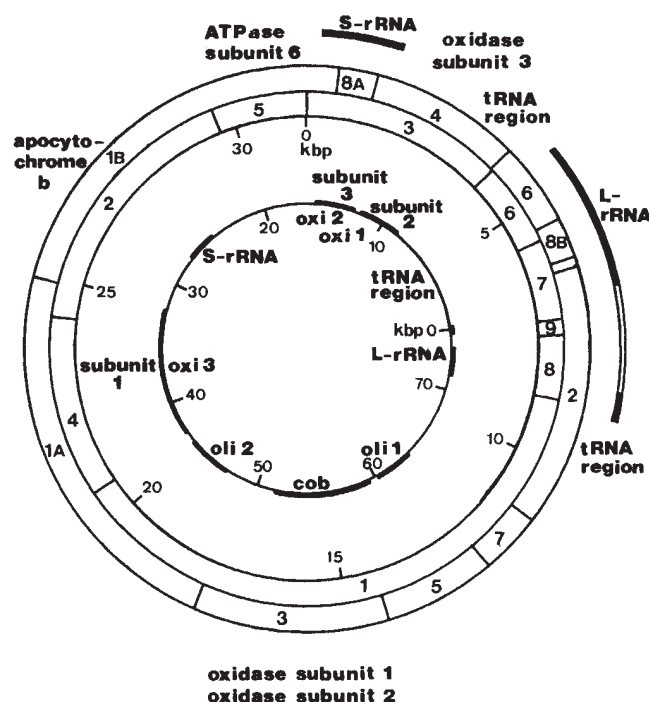


Fig. 2 Restriction fragments used as hybridization probes. The *petite* mutant strains used are named according to the yeast gene that they contain; they were DS200A1 (*oxi*1)², DS40 (*oxi*2)²⁴, DS6/A407 (*oxi*3) (unpublished), DS400/A12 (*cob*)²⁵ and DS14 (*oli*2)³. The bar above the line indicates the position of coding regions of the gene in each case; the numbers indicate base pairs starting from the NH₂-terminal coding end. Arrows show the direction in which NH₂ or COOH terminal-coding regions would lie if they were in the *petite*. The *Hinf*I–*Taq*I *oxi*3 ([*oxi*A]) probe contains only exon 5, which is towards the promoter-distal end of the *oxi*3 split gene. The *oxi*1 ([*oxi*B]) probe corresponds essentially to the promoter-proximal coding half of the gene, the *oxi*2 ([*oxi*C]) probe to the promoter-distal half of the gene. The *cob* gene probe is part of the first exon of this split gene and the *oli*2 ([*oxi*A]) probe corresponds to the promoter-distal two-thirds of this gene. All *petites* have been completely sequenced^{2,3,24,25}. The solid bar below the line gives the position and length in base pairs of the restriction fragment used as a probe. Other symbols show the position of restriction endonuclease recognition sites. ▲, *Hinf*I; ★, *Hind*III; ↑, *Taq*I; ●, *Mbo*I; ■, *Eco*RI; bp, base pairs.

In yeast, mutations in two mitochondrial genes can give rise to an oligomycin-resistant phenotype. Both genes code for components of the ATPase complex; the *oli1* gene codes for the so-called DCCD-binding protein⁹, the *oli2* gene for subunit VI of the ATPase complex. Previous genetic work on oligomycin resistance in *Aspergillus* has shown that one gene maps in the mitochondrial genome (*[oliA]*)¹⁰; the *[oliB]* group of mutations is probably part of *[oliA]* and one in the nuclear genome (*[oliC]*). Figure 1 shows that a yeast *oli2* gene probe hybridizes to *Hind*III fragment 5 of *Aspergillus* mtDNA and to *Hae*III fragment 1A or 1B (presumably to 1B). An *Aspergillus* mitochondrial gene thus corresponds to the *oli2* gene of yeast, maps between the *[cobA]* gene and the small rRNA and probably corresponds to the *[oliA]* gene.

The overall plan of the mitochondrial genome of *Aspergillus nidulans* has some similarity to that of *S. cerevisiae*, although they differ considerably in size (*A. nidulans* 31.5 kilobase pairs, *S. cerevisiae* 75 kilobase pairs) and gene arrangement. In both organisms the mitochondrial rRNA genes are not contiguous, and the large rRNA gene is split by an intron¹⁷. The space between them is very large (25 kilobase pairs) in *S. cerevisiae*, and contains the *oxi1* and *oxi2* genes and a cluster of tRNA genes¹, whereas in *A. nidulans* they are separated by only 2.5 kilobase pairs containing the [*oxiC*] (= *oxi2*) gene and part of the tRNA gene cluster. The two rRNA genes, [*oxiC*] (= *oxi2*), [*cobA*] (= *cob*) and [*oliA*] (= *oli2*) have the same positions relative to one another in the two organisms, although the distances between them are quite different. The *Aspergillus* equivalent of the yeast mitochondrial *oli1* gene is in the nucleus¹⁸. The [*oxiA*] (= *oxi3*) and [*oxiB*] (= *oxi1*) genes are in the mitochondrial genome in both organisms, but in very different positions. The coding sequences of all five genes must be remarkably strongly conserved to show such clear hybridization specificity. In coding regions that have been sequenced, up to 60% of the amino acids and 72% of the base pairs are the same in *S. cerevisiae* and *A. nidulans* (R.W.D. *et al.*, unpublished).

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The hybridization results are summarized in Fig. 3. Previous information about the nature and relative positions of *Aspergillus* mitochondrial genes has come from recombinational mapping^{11,12} of mitochondrially inherited mutations¹³. Five classes of such mutations are known in *Aspergillus*: [*oliA*]¹⁰, conferring oligomycin resistance, [*camA*] and [*camB*] (probably in the same gene), conferring chloramphenicol resistance^{7,14} [*cs-67*], which is a cold-sensitive mutation¹⁵ and [*sumD*]¹⁶.

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Comparison of benzo(a)pyrene metabolism and sister chromatid exchange induction in mice

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Genetic differences in the inducible arylhydrocarbon hydroxylase (EC 1.14.14.2) (AHH) system, which is involved in the multi-step metabolism of hydrocarbons, are known to exist in both humans and mice¹⁻⁶. However, the predictive value of AHH activity in human or murine tissues, assayed as benzo(a)pyrene hydroxylation, as an index of individual susceptibility to mutagens and carcinogens, remains unclear⁵⁻⁸ because of apparent inconsistencies between results obtained from different *in vitro* and *in vivo* systems. This situation may in part reflect the complexity of the pathways involved in drug metabolism, which combine both activation and detoxification. To determine the relationship of metabolic potential to an easily quantified, short-term *in vivo* end point of genetic damage, we compared the ability of AHH inducible and uninducible mice to metabolize a procarcinogen, benzo(a)pyrene (BP), with the *in vivo* induction by BP of sister chromatid exchanges (SCEs). SCE induction has been shown to correlate with mutagenesis^{9,10}. We report here that although BP did cause an increase in SCEs in test animals, the extent of this increase did not differ between the inducible C57BL/6 mice and the uninducible DBA/2 mice. Moreover, prior exposure to an AHH inducer, 3-methylcholanthrene (3-MC), did not increase the number of BP-induced SCEs in C57BL/6 mice. This lack of correlation between benzo(a)pyrene hydroxylase (BP-OH) inducibility and SCE response reinforces the idea that other metabolic steps, such as detoxification or DNA repair, may influence the overall genetic impact of a drug.

Mice were examined for their ability to convert BP to the fluorescent product 3-hydroxybenzo(a)pyrene, as a measure of the early metabolism of this drug by the AHH system¹¹ (Fig. 1). C57BL/6 was used as the inducible strain as these mice show BP-OH induction in both the liver (Fig. 1a, b) and bone marrow (Fig. 1c) in response to prior administration of 3-MC. DBA/2 was used as the uninducible strain as these mice showed no hepatic enzyme induction (Fig. 1d, e) in response to 3-MC and only minimal enzyme induction in peripheral tissues (Fig. 1f).

The same mouse strains were also tested for epoxide hydrolase (EC 4.2.1.63) activity, the second enzyme in the proposed metabolic pathway of BP to its ultimate carcinogenic form¹² (Table 1). There was no significant difference in epoxide hydrolase activity between the two strains, and neither strain showed 3-MC inducibility for this enzyme.

Having confirmed differences in AHH activity between the two strains, these mice were used for *in vivo* SCE analysis following partial hepatectomy as previously described¹³ with or without prior 3-MC induction. BP-induced SCEs were scored in both liver and bone marrow cells and the results from multiple animals pooled (Fig. 2). The data were compared by a two-tailed *t*-test which took into account differences in baseline SCE frequencies.

Although prior 3-MC treatment produced a 6.8-fold increase in BP-OH activity in regenerating liver of the inducible C57BL/6 mice (Fig. 1b), this pretreatment did not alter the number of BP-induced SCEs in either the liver or bone marrow of this strain (Fig. 2a, b). A small increase in baseline SCE frequencies was observed with induction, attributable to the 3-MC itself, which is metabolized by the AHH system and known to induce SCEs when activated¹⁴. However, there was no statistically significant difference in BP-induced SCEs above this

baseline effect with and without prior BP-OH induction by 3-MC.

Both the inducible and uninducible strains, in the absence of enzyme induction, showed fairly similar SCE induction in liver cells in response to BP (Fig. 2a, c; $P > 0.1$ at 50 and 200 mg per kg BP), although the DBA mice showed a slightly larger SCE response to BP in bone marrow (Fig. 2b, d; $P < 0.01$) than did C57BL/6 mice. Our previous observation that reduced liver function can lead to an enhanced response to clastogens in peripheral tissues¹³ may account for this effect, as well as the observation of Nebert¹⁵ that nonresponsive mouse strains exhibit larger drug-induced effects in distant tissues than do responsive mice.

In contrast to the results in BP, the induction of SCEs in response to 3-MC differed markedly between the two mouse strains. In C57BL/6 mice, 3-MC caused only a small increase in SCE frequencies (from 6.2 to 8.7 SCEs per cell, Fig. 2a, b), but effected a marked increase in enzyme activity (Fig. 1b, c). In contrast, in the DBA/2 mice, 3-MC does not induce AHH activity, but it produces a large increase in SCE frequencies (from 5.1 to 15.9 SCEs per cell, Fig. 2c, d), even 2 days after its administration. This result may be due to the prolonged persistence of 3-MC in the strain with the lower metabolic capabilities.

The inability of BP to induce additional SCEs over the heightened baseline level in 3-MC-treated DBA mice (Fig. 2c, d) remains unexplained. It does not reflect a saturation of the SCE response, because higher levels of SCEs per cell can be obtained with other drugs^{13,16}. The lack of additional SCE induction might instead reflect some form of competition (between the two hydrocarbons, 3-MC and BP), perhaps for limited metabolic enzymes or the cytoplasmic receptor known to be deficient in DBA mice¹⁵.

The metabolism of BP can involve multiple pathways leading to a variety of products, many of which are capable of reacting with DNA. 3-MC treatment of inducible mouse strains has been reported to induce a distinct class of cytochromes that activate hydrocarbons such as BP to their ultimate carcinogenic form, in this case the 7,8-diol-9,10 epoxide¹⁷. In the present

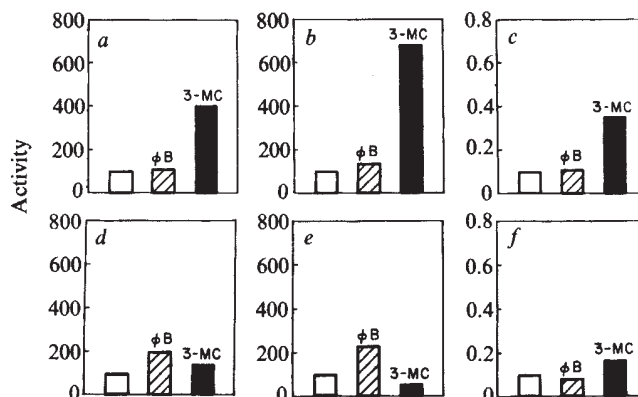


Fig. 1 Benzo(a)pyrene hydroxylase activity in intact liver (a, d), regenerating liver (b, e) and bone marrow (c, f) of C57BL/6 (a, b, c) and DBA/2 (d, e, f) mice. Activity expressed as percentage of control activity (open bars) seen with either phenobarbital (PB) (hatched bars) or 3-methylcholanthrene (3-MC) (solid bar) induction. Hepatectomy, when done, immediately preceded induction. Bone marrow was collected from non-hepatectomized mice. Animals received either three daily injections of 80 mg phenobarbital (Sigma) per kg in 8% ethanol or 80 mg 3-MC (Sigma) per kg in corn oil. Controls received no injection. Tissue was collected at 72 h after induction from PB-treated animals, at 50 h from 3-MC-treated animals and 67 h from control animals (times reported to be maximal for enzyme induction)²⁸⁻³⁰. Livers were homogenized and bone marrow cells sonicated in 0.15 M KCl and the 9,000g supernatant (S-9) material used in the enzyme assays. Liver S-9 was incubated for 5 min and bone marrow for 20 min in 56 mM Tris, 2.8 mM MgCl₂, 3.7 × 10⁻⁴ M NADPH, 4.3 × 10⁻⁴ M NADH, 0.6 mg ml⁻¹ albumin with 8.5 × 10⁻⁵ M BP ($\epsilon_{384.5} = 27,542$ in methanol). The reaction was stopped with hexane/acetone (3.75:1 v/v). After mixing, the organic phase was extracted with 1 M NaOH and the fluorescence read with a Perkin-Elmer MPF-3 (excitation 396 nm; emission 522 nm)¹¹. This fluorescence was compared with that of pure 3-hydroxybenzo(a)pyrene ($\epsilon_{380} = 36,300$ in alcohol) and activity expressed as pmol 3-OH-BP formed per mg S-9 per min. Bone marrow activity was 1/1,000th of that in liver.

Table 1 Epoxide hydrazase activity in regenerating mouse liver

Induction	C57BL/6	DBA/2
None	245 ± 72	243 ± 80
Phenobarbital	523 ± 41	354 ± 138
3-Methylcholanthrene	292 ± 81	262 ± 109

Activity is expressed as pmol ^3H -styrene glycol formed per min per mg S-9, measured by the conversion of ^3H -styrene oxide (Amersham, specific activity 88 mCi mmol $^{-1}$) to styrene glycol²⁷. Approximately 2.5 mg liver S-9 was incubated with 125 mM Tris (pH 9.0, with 0.025% Tween) and 1 mM styrene oxide (diluted with cold reagent to 190,000 c.p.m.) for 30 and 60 min. After two extractions with *n*-hexane, the reagent mixture was extracted with 2 ml ethyl acetate and 200- μl aliquots counted in a Beckman LS8000 scintillation counter. Each value represents the mean of four trials $\pm$ s.d.

experiments, the number of SCEs induced by BP *in vivo* was independent of the AHH inducibility of the mouse strain used or the exposure of inducible mice to 3-MC. The lack of correlation between AHH inducibility and SCE induction suggests either that constitutive AHH activities and products thus formed are responsible for the SCE induction observed or that the involvement of other enzymes in the metabolic pathway, such as epoxide hydrazase, obscures any differences which may exist in the AHH system.

This lack of correlation between metabolic potential expressed as AHH activity and biological end points has been observed previously. For example, no relationship was observed between AHH inducibility and the *in vitro* induction of SCEs by BP¹⁸, the activation of BP by S-9 fractions to a mutagen in the Ames test^{19,20}, the induction by hydrocarbons of tumorigenesis in humans⁶ or in mice⁸, or the extent of covalent binding of BP metabolites to DNA²¹. Several of these effects can, however, be enhanced by inhibiting epoxide hydrazase activity^{22,23}, suggesting that this enzyme, which shows no variability between the two strains examined, may have a critical role. Developmental differences in epoxide hydrazase levels²⁴ or in other metabolic enzymes²⁵ may explain a recent study²⁶ of the effect of BP on isolated early mouse embryos, in which AHH activity was not measurable, and a larger SCE increment was observed in inducible strains than in noninducible strains.

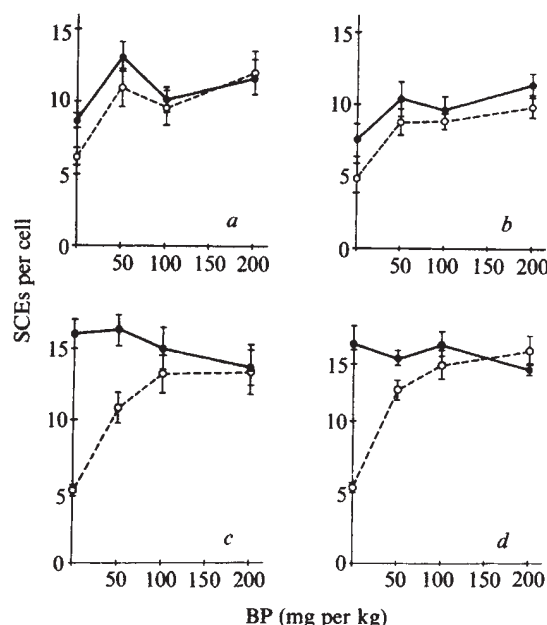


Fig. 2 Induction of SCEs by benzo(a)pyrene (BP) in the liver (a, c) and bone marrow (b, d) of partially hepatectomized (two-thirds removal of liver) C57BL/6 (a, b) and DBA/2 (c, d) young (7–9 weeks) female mice (Charles River) with (solid line) and without (broken line) prior 3-MC treatment (as in Fig. 1). Animals received BP by intraperitoneal injection (53 h post-hepatectomy) followed by a series of 10^{-2} M bromodeoxyuridine injections. The following day the animals received two 0.4-ml injections of colchicine (0.2 mg ml^{-1}) and liver and bone marrow cells were collected from each animal. Each point on the graph represents the mean of three to six animals (a minimum of 20 cells was examined per animal) and the error bars indicate s.e.

The inability to demonstrate clear and consistent relationships between AHH inducibility and a variety of genetic responses, including SCE induction, suggests that other biological pathways including alternative activation and detoxification, and perhaps DNA repair, influence the end points obtained. The complexity of steps involved in reaching a biological end point thus makes measurement of a single enzyme in the metabolic pathway unreliable as a predictor of an individual's susceptibility to the *in vivo* effects of a procarcinogen.

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Chemical cross-linking restrictions on models for the molecular organization of the collagen fibre

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The nature of the precise packing of collagen molecules into a collagen fibril, producing the characteristic regular banding, is still debatable. The problem has been approached using electron microscopy and X-ray diffraction techniques, and several models have been proposed, including hexagonal packing, an octafibrillar structure, a two-strand rope and a five-strand rope (for review, see ref. 1). For the past decade the pentafibrillar model, originally proposed by Smith², has been widely accepted as the fundamental building unit. This model, based on the quarter-stagger end-overlap hypothesis of Hodge and Petruska³, was

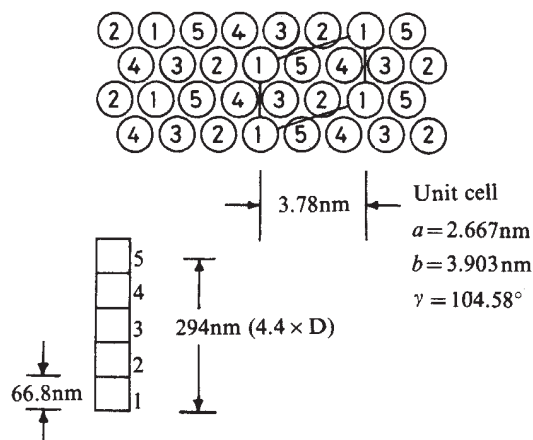


Fig. 1 Projection of the hexagonally packed collagen molecules along their axis. The small distortions of the lattice⁷ and the slight (4°) tilting of the collagen molecules are not shown. The numbers 1–5 refer to the molecular segments (see lower left) in one 66.8-nm (D) thick transverse section. The packing arrangement may be considered as horizontal layers of the quarter-stagger end-overlap³ system, running 1 to 5 from right to left, with successive layers displaced from left to right by 3.78 nm, thus generating the unit cell⁵ outlined.

supported by the X-ray diffraction data of Miller and Wray⁴. These X-ray data have now been reinterpreted by Hulmes and Miller⁵ in terms of a quasi-hexagonal packing of collagen molecules. We argue here that until other characteristic parameters are taken into account, in particular the chemical cross-linking evidence, the packing problem is still unresolved.

The recently proposed model⁵ involves the juxtaposition of the collagen molecules in a slightly distorted two-dimensional hexagonal lattice, having a specific tilt of about 4° between the collagen molecules and the normal to the basal lattice plane, together with some longitudinal shear. The model is a development of the packing arrangement proposed by McFarlane⁶. The apparent advantage of this model is that it provides a better fit with the X-ray diffraction data compared with other models. Note that both models (the penta-fibrillar rope and the quasi-hexagonal arrangement) rely essentially on the same X-ray diffraction data.

These three-dimensional structures must incorporate the quarter-stagger end-overlap array³ to generate the banding pattern observed by electron microscopy. However, none of these models considers the characteristic chemical cross-linking evidence of collagen. In particular, the new quasi-hexagonal model does not account for the restrictions on proposed molecular arrangements imposed by the known location of the intermolecular cross-links.

A cross-section through the proposed quasi-hexagonal model perpendicular to the collagen fibrillar axis is shown schematically in Fig. 1. The numbers 1–5 represent specific distances along the collagen molecules commensurate with the D-stagger in the end-overlap model³. To fit the basic features of the X-ray diffraction pattern⁴, Hulmes and Miller⁵ stagger successive layers laterally (see Fig. 1) by 3.78 nm relative to each other, generating the 3.78-nm row lines observed in the X-ray fibre diffraction patterns⁴ and developing a two-dimensional lattice defined by $a = 2.607$ nm, $b = 3.903$ nm with $\gamma = 104.58^\circ$ (Fig. 1).

The location of the bifunctional cross-links has been established by biochemical techniques for the fibrous types of collagen, type I^{7,8}, type II^{9,10} and type III^{11,12}, as only occurring between the telopeptide lysine-aldehyde and the ϵ -NH₂ group of specific lysine or hydroxylysine residues 25 nm from either end of the molecule, that is, the end of the overlap region (Fig. 2a). We have now extended these observations to mature tissue and have demonstrated that cross-linking occurs through polymeric interactions involving the C-terminal non-helical

region of $\alpha 1CB6$ and the helical peptide $\alpha 1CB5$. This polymeric cross-linked material, poly- $\alpha 1CB6$, has been isolated from mature bovine tendon and partially characterized^{13,14}. The important factor relating to the molecular structure of the fibres is that this cross-linking arrangement can only occur if the C-terminal ends of the molecule are in register. In terms of the numerical notation of the D-stagger of the molecules, the $\alpha 1CB6$ - $\alpha 1CB5$ dipeptide is classified as 1, 5—the molecules align in the lattice such that 1's are adjacent to 5's and vice versa. In the fibre structure, individual collagen molecules must experience neighbours staggered by D and have at least one neighbour aligned in longitudinal register to generate limited interconnections of the type 1, 5; 1, 5; 1, 5 within the fibre, thereby generating poly- $\alpha 1CB6$ (Fig. 2b).

Analysis of the new structure proposed by Hulmes and Miller⁵ shows that such a cross-link scheme is unattainable. Furthermore, if to accommodate this packing restriction the molecules in the structure are moved longitudinally or laterally, then the proposed unit cell on which the X-ray argument is based will have to change. This model must therefore be adapted to fit the cross-linking data. This can readily be achieved if C-terminals in successive layers are juxtaposed with reducible cross-links between $\alpha 1CB6$ and $\alpha 1CB5$ (or 1, 5 in quarter-stagger nomenclature) to form a polymer containing 1:1 proportions of $\alpha 1CB6$ and $\alpha 1CB5$. Analysis of poly- $\alpha 1CB6$ did indeed show an approximate 1:1 ratio of these two peptides¹⁴. Hence, by using the cross-linking data to modify the model suggested by the physical data, a detailed and more accurate quasi-hexagonal model could be postulated.

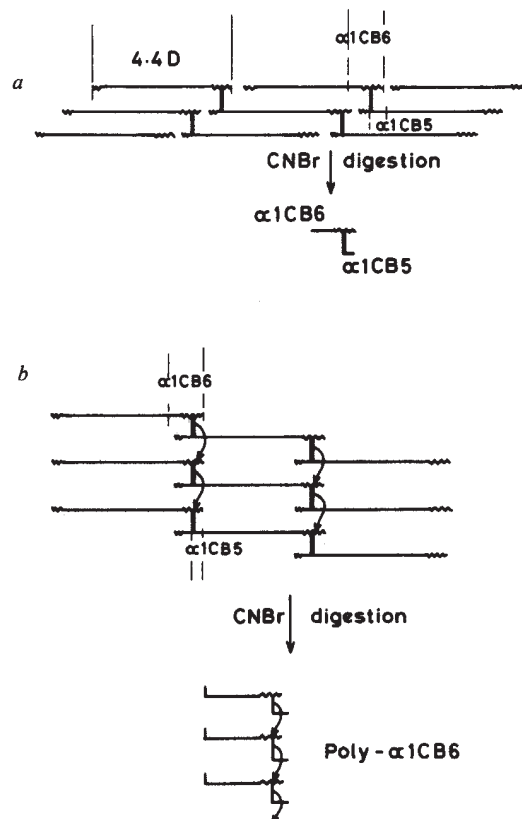


Fig. 2 a, Schematic quarter-staggered array of collagen molecules showing the location of a reducible cross-link between $\alpha 1CB6$ and $\alpha 1CB5$ peptides and the resultant 'H' peptide derived from a cyanogen bromide digest. b, Diagrammatic representation of the proposed formation of poly- $\alpha 1CB6$. Layers of quarter-staggered collagen molecules are shown in register, that is, layer 1 is in register with layer 2 which is in register with layer 3. Each reducible cross-link between $\alpha 1CB6$ and $\alpha 1CB5$ is aligned and closely packed with a reactive residue in the adjacent $\alpha 1CB6$ peptide in the molecule in the next layer, thus several layers can be interlinked by repeating this simple additive process.

Recent evidence on variations in fibre size from electron microscopy has suggested that there is a basic building fibril of about 8.0 nm¹⁵. Similarly, the X-ray evidence of Nemetschek¹⁶ using stained fibres provides strong evidence for a unit cell of 8.0 nm, contrasting with the 4.0-nm cell of the other penta-fibril and hexagonal models. These data have received recent support from the electron diffraction studies of Squire and Freundlich¹⁷. Fraser's group suggests¹⁸ that four penta-fibrils align in register to form such an 8.0-nm unit, which they term the tetragonal cell or unit. Considering the restrictions that such a structure puts on the possible extent of formation of polymeric cross-linked material, this model similarly, in its simplest form, cannot readily be adapted to fit the chemical evidence. Intrapenta-fibrillar reducible cross-links can be formed relatively easily so that the unit itself is stabilized. However, when these cross-links are stabilized by addition reactions with other aligned C-terminals in other penta-fibrils, only internal interactions within the tetragonal unit seem possible due to the large interatomic distances between sites in different units. This would seem to limit the maximum molecular weight of the resultant polymeric cross-linked material to a much lower range than that actually observed. A further difficulty with this model concerns the discrepancy between observed and calculated densities, as discussed by Hulmes and Miller⁵.

It might be profitable to view the hexagonal models^{6,5} as being built up from groups of five collagen molecules (or multiples as favoured by the larger unit cells suggested by the more recent X-ray evidence of Nemetschek¹⁶), each lying on a hexagonal lattice site (or a slightly distorted hexagonal lattice in the final analysis) so that they form a collapsed penta-fibril which in turn aligns with other such units to complete a full two-dimensional array. Certainly, between the extremes of the McFarlane model⁶ and the Hulmes and Miller model⁵, there might be scope for organizing limited runs of 1, 5 interconnections.

The parameters which govern the organization of such interactions may be determined by considering the precise restrictions imposed on the structure of the collagen fibril by the known characteristics of poly- α 1CB6 as follows. (1) The bifunctional cross-links formed between staggered molecules to give the 1, 5 interactions must limit the movement of the non-helical region involved. (2) Chemical evidence strongly suggests that the stable cross-link is produced by an addition reaction of a reactive group in an adjacent molecule with the bifunctional cross-link. An exogenous molecule (such as a lipid) could be involved as a chemical bridge in this reaction, in which case the intermolecular distances of the collagen molecules need not be restricted. However, no evidence exists for such a mechanism and, given the precise collagen molecule packing, this pathway is extremely unlikely. The most feasible mechanism would involve a reactive side chain in the nearest-neighbour collagen molecule. This prediction limits the possible intermolecular distance to a maximum of the length of the longest amino acid side chain. (3) The experimental data suggest that the range of poly- α 1CB6 polymers characterized would be formed from the interactions of molecules in 5–10 layers of close-packed collagen sheets.

Clearly, the rigorous application of the chemical evidence can profoundly affect the types of model constructed to explain the physical data. Model building is of considerable value but has severe limitations, particularly if all lines of evidence are not accounted for. Definitive chemical evidence on cross-linking is now available and should be regarded as at least as important as the physical data and may, indeed, ultimately prove to be the most crucial criterion for unravelling higher molecular organization in the collagen matrix.

After submission of this paper various arrangements of the collapsed penta-fibril model similar to that proposed by us was postulated by Trus and Piez¹⁹, but without consideration of the chemical cross-linking evidence.

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Direct observation of a transverse periodicity in collagen fibrils

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Collagen fibrils have been extensively studied by electron microscopy, but only scant evidence has yet been obtained about their transverse structure. A transverse periodicity of about 40 or 80 Å might be expected if the microfibril model (until recently the common preference) is correct^{1–9}, but little direct evidence for such a periodicity has been obtained so far. We have studied electron micrographs of longitudinal cryosections of rat tail tendon. These appear very fibrous when studied by eye, but they produce at best only weak diffuse equatorial peaks in optical diffraction patterns. Nevertheless, we show here that like other fibrous protein structures, they do possess a strong transverse periodicity as revealed by their self-convolution functions (or auto-correlation functions)^{10–12}. The observed periodicity is consistent with the presence of an 80 Å structural unit in collagen fibrils *in vivo*.

Many aspects of the structure of collagen fibrils have been satisfactorily accounted for in recent years. The chain conformation in tropocollagen molecules has recently been refined by Fraser *et al.*¹³, and the axial striation patterns in positively stained aggregates of collagen have been correlated well with the known amino acid sequence of the α 1 collagen chains^{14–21}. The sequence studies show that the characteristic *D*-period of about 670 Å in collagen fibrils is due to the 670 Å axial stagger between molecules and that this stagger, or a multiple of it, is such that the number of polar and apolar interactions between adjacent molecules is maximized.

However, considerable controversy remains about the arrangement of collagen molecules in a transverse view of a collagen fibril. For several years the favoured model was one in which fibrils were formed from an ordered near-tetragonal array of microfibrils of diameter ~38 Å and probably containing five strands of tropocollagen molecules. Four such microfibrils, each twisted to have 4₁ helical symmetry, would then be related by an approximate 4₃ screw axis to give a repeating unit about 80 Å square^{1,3,6,9}. Other published models also have microfibrils 38 Å in diameter but containing differing numbers of strands^{4,5,7,8}. In support of such structures there have been several observations of individual filamentous elements about 40 Å in diameter in electron micrographs of collagen preparations (see refs 22 and 23). In addition, Parry *et al.*^{24,25} have recently reported that in many types of connective tissue the fibril diameters are always multiples of about 80 Å; a result clearly favouring the existence of an 80 Å structural unit.

But a radically different model for collagen structure has recently been proposed by Hulmes and Miller^{26,27}. This model explains the observed X-ray diffraction data on collagen² not in terms of a near-tetragonal lattice of microfibrils, but rather in

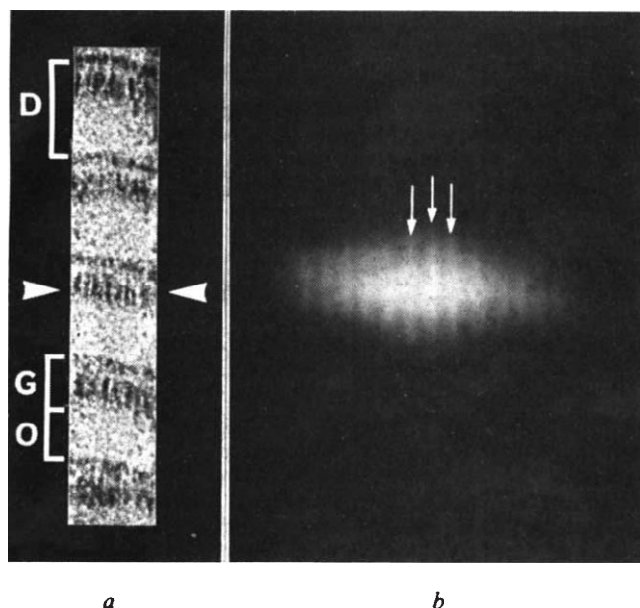


Fig. 1 *a*, Electron micrograph of part of a negatively stained, fixed collagen fibril from rat tail tendon (fibril axis vertical) showing the characteristic pattern of alternating dark (stain filled) and light (stain excluding) bands which together define one *D*-period (*D*). In molecular terms the dark band (*G*) is the gap region in the modified quarter-stagger model of Hodge and Petruska³⁸, and the light band (*O*) is the region of molecular overlap. Within the dark band between the arrowheads thin vertical strands of protein (white) can be seen. The self-convolution function of this single dark band is shown in *b*. As indicated by the arrows, regularly spaced vertical white lines can be seen in the convolution indicating that the strands in *a* have a well-defined average lateral separation. In *a*, *D* is 708 Å and in *b*, *s* (the lateral period between adjacent arrowed lines) is 55 to 60 Å. Note that the convolution *b* is enlarged relative to *a*.

terms of a molecular crystal²⁸ in which straight collagen molecules, somewhat tilted to the fibril axis, pack in a quasi-hexagonal lattice²⁹. Miller²⁷ has shown that such a model can account well for the intensity distribution in the equatorial and near-equatorial regions of the observed X-ray diffraction pattern and it explains some features not accounted for by the tetragonal lattice.

It is by no means clear, however, that this new model could explain the 80 Å increments in fibril diameter observed by Parry *et al.*²⁴⁻²⁵. On the other hand, one might expect from these observed diameters that it should be possible to see an 80 Å lateral periodicity in electron micrographs of collagen fibrils. But only in one reported case³⁰ have sections of collagen fibrils revealed the presence of an 80 Å periodicity. The fibrils, which were very different in character from those in tendon, were from the inner layers of the dogfish egg capsule and they showed a square lattice of side 80 Å in transverse sections. No such structure has yet been reported in connective tissue from higher vertebrates.

In an attempt to study the transverse structure in rat tail tendon, we are using the relatively novel cryosectioning technique, which we have successfully applied to muscle^{31,32}, to obtain both transverse and longitudinal frozen sections of tendon. Briefly, ultra-thin longitudinal sections, which are not as difficult to obtain as transverse cryosections, have already provided direct evidence for a prominent transverse periodicity larger than the unit cell dimensions in the hexagonal model of Hulmes and Miller²⁶ but compatible with the presence of an 80 Å structural unit.

Fresh rat tail tendon was fixed for 60 min in 2.5% glutaraldehyde at 4 °C, soaked for 45 min in 30% glycerol in water (which

acts as an antifreeze agent), dissected into small pieces (1 mm cube at most) and frozen rapidly by immersion in Freon 12 cooled by liquid nitrogen. The frozen tissue, held at about -110 °C, was sectioned, using an LKB ultramicrotome and Cryokit, with a glass knife at -45 °C, onto a trough containing 50% dimethylsulphoxide. Sections or section fragments (sections tended to tear because of the very fibrous nature of the tendon) were collected on formvar/carbon coated grids and either negatively stained with 2% ammonium molybdate or positively stained with 2% uranyl acetate and Reynolds lead citrate. In some preparations the initial fixation step in glutaraldehyde was omitted.

Electron micrographs of these various preparations often showed remarkably clear structure (as in Fig. 1*a*); they were comparable to or better than the best published micrographs of negatively stained or positively stained isolated fibrils obtained by homogenization (see for example refs 33 and 34). Stereo-pair electron micrographs also showed that in many of these preparations only surface negative staining was present and in such cases, despite the low inherent contrast, there was a considerable enhancement of detail and clarity. In addition to the marked *D*-periodicity along these fibrils (*D* in Fig. 1*a*) many seemed to show some evidence of transverse structure. They were frequently very fibrous in appearance, as noted previously by others^{33,34}, but optical diffraction analysis showed little tangible trace of any equatorial spacings (other than diffuse and

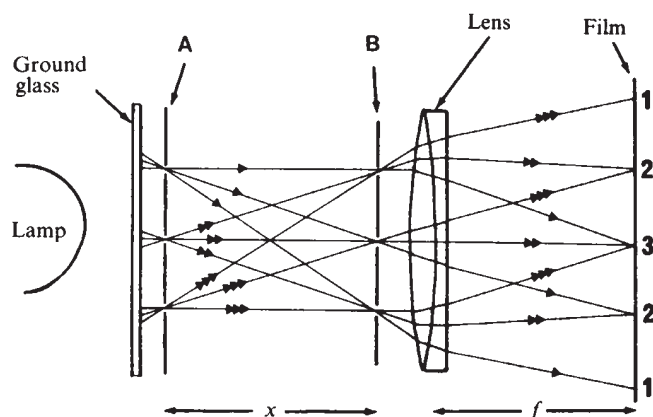


Fig. 2 Schematic ray diagram of the convolution camera^{10,11}. The lamp and ground glass (or opal glass) screen provide an extended diffuse light source which evenly illuminates a transparency (*A*) of the micrograph to be studied. An identical transparency (*B*) at a distance *x* from *A* is positioned close to a lens which is set to focus parallel light in the plane of the film (right). The film is conveniently held in a reflex camera body. While viewing the image in the focal plane using the camera viewfinder, the transparency *B* is rotated in its own plane until the images in *A* and *B* are exactly parallel. This can be judged by making the central spot in the image plane as small and symmetrical as possible. When this has been done, the image is the self-convolution of the micrograph in *A* (or *B*). The ray diagram illustrates the light paths for a model micrograph containing three equidistant transparent objects (here shown as holes). Each hole in *B* acts as a pinhole camera and transmits light from *A* to form an inverted image of *A* in the film plane. But since the holes in *B* are periodically displaced, so are the images of *A* in the film plane. It will be seen that the central feature in this plane has light reaching it from each hole in *A*; it is an average of the features in *A*. Similarly further rays reach the film on each side of the centre and are separated from the centre by the periodicity in *A* (or *B*). Thus the self-convolution is a particular form of average of the structure in *A* (or *B*) and any regularities in the micrograph tend to be enhanced in the self-convolution. If *x* = *f* then the convolution has the same magnification as the micrograph, otherwise the magnification is *f*/*x*. Note that *f* is the focal length of the lens and must be fixed, whereas *x* can be varied at will. Full details of the convolution technique will be given elsewhere (ref. 12 and manuscript in preparation).

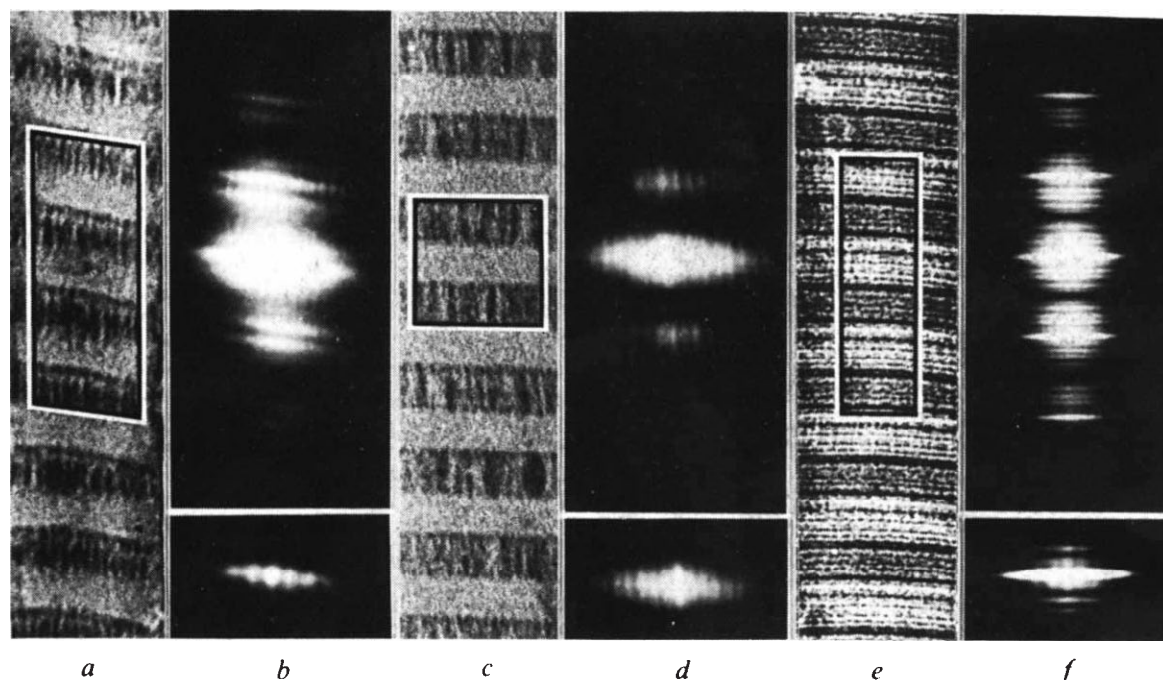


Fig. 3 *a*, *c* and *e* are electron micrographs of collagen fibrils which were *a* fixed and negatively stained; *b*, unfixed and negatively stained and *e*, fixed and positively stained. The self-convolution functions of the outlined areas are shown in *b*, *d* and *f* respectively. In each case the whole self-convolution is shown at the top and just the central region is shown at the bottom. In *b* and *f*, the lower prints are at twice the magnification of the upper prints. Note that the whole outlined areas in *a* and *e* were used to give *b* and *f*, but that in *c*, the central light band in the outlined area on the convolution negative was blacked out using Indian ink (without doing this the light from the highly transparent but, in negatively stained preparations, relatively structureless overlap regions tends to swamp the convolution and to obscure the features of interest in the gap regions). The convolution in *d* therefore reveals the structure of the dark bands only. Clear lateral periods can be seen in *b*, *d* and *f* and the spacings measured from these were respectively, $s = 75\text{--}80\text{ \AA}$ ($D = 583\text{ \AA}$), $s = 95\text{--}100\text{ \AA}$ ($D = 625\text{ \AA}$) and $s = 55\text{--}60\text{ \AA}$ ($D = 598\text{ \AA}$).

unmeasurable regions barely perceptible above the noise level) out to a reciprocal spacing of about $1/20\text{ \AA}^{-1}$. However, similar negative results using optical diffraction have been obtained from other fibrous protein structures such as isolated paramyosin filaments¹⁰ and synthetic tactoids of tropomyosin¹¹. In both cases some trace of a transverse periodicity seemed to be apparent from visual inspection, and despite the failure of the optical diffraction method, the existence of a strong lateral periodicity (about 35 to 40 $\text{\AA}$ in each case) was confirmed by means of the so-called convolution camera^{10,11}. In this camera (Fig. 2) the self-convolution function (or auto-correlation function) of an image is generated by a simple optical system¹⁰⁻¹² comprising a diffuse light source, two identical negative transparencies (A and B) of the image to be analysed, a lens and a film holder. If periodicities are present in the micrograph, then they will become very much enhanced in the self-convolution. The extent of the order can also be deduced. Just like the Patterson function in X-ray crystallography, to which it is analogous, the self-convolution function reveals the presence of strong inter-object vectors in the image being studied.

Application of the self-convolution technique to collagen fibrils such as that in Fig. 1*a* has revealed directly that a strong transverse periodicity is often present. Other examples are shown in Fig. 3. The spacing of the periodicity was in each case related to the D -period of the same fibril which in turn was deduced from the known calibration of the electron microscope. It was found that the D -spacing varied between different preparations and even between different fibrils in the same electron micrograph, the observed D -spacings generally being in the range 580–710 $\text{\AA}$. A wide variation of this period as a function of moisture content has previously been observed in X-ray diffraction studies of tendon³⁵ and presumably this behaviour is

being mimicked here by a variable stain content in our preparations.

It can be seen in the self-convolution in Fig. 1*b* that there are strong regularly spaced lines of density (white) running parallel to the fibril axis in Fig. 1*a*. Similarly in Fig. 3*b*, *d* and *f* it is clear that the lines running from left to right (that is, perpendicular to the fibril axis) are composed of discrete blobs of density (white) with a fairly uniform spacing. These appearances are direct evidence that there is regular transverse structure within the fibrils which gave rise to the convolutions. However it was found that not all fibrils or even all areas of the same fibril gave equally regular convolution patterns. But, whenever the lateral periodicity could be seen clearly, it always had a measured spacing (s) of about the same size. Most of the fibrils which were studied were from fixed and negatively stained preparations (for example Figs 1*a* and 3*a*) and the observed range of side-spacing was $s = 55\text{--}80\text{ \AA}$. Preliminary examination of unfixed fibrils (Fig. 3*c*) or fixed positively stained fibrils (Fig. 3*e*) has given s values of about 90–100 $\text{\AA}$ and 55–60 $\text{\AA}$ respectively.

Despite the apparent lack of order in some fibrils, it is clear in others (for example Fig. 3*a*) that the order is very good. In some cases the transverse order has been seen up to eight to ten s periods from the centre of the convolution, indicating a correlation of lateral structure over a range of about 500 to 800 $\text{\AA}$. This would represent most or all of the width of many collagen fibrils^{24,25}. Similarly the presence of obvious structure along the strong transverse lines above and below the central line through a convolution such as Fig. 3*b*, shows that the lateral structure in one D -period correlates well with that in the adjacent D -periods along the fibril. We have observed similar correlation over an axial range of at least four D -periods, indicating that in some preparations there is a very high degree of structural regularity

along the fibrils. It is not yet clear whether the observed differences in regularity within or between fibrils are a result of the preparative procedure, or are an inherent feature of the fibrils *in vivo*.

The observation of a transverse periodicity of 55–100 Å implies that there is some form of molecular organization of intermediate size within collagen fibrils; this, clearly, is incompatible with the model of Hulmes and Miller²⁶ as it stands. Recently Bailey *et al.*³⁶ have also questioned the validity of this model on the basis of cross-linking data. On the other hand our results tie in well with the observations of Parry *et al.*^{24,25} on fibril diameters and they are consistent with the presence of an 80 Å molecular aggregate. However, it remains to be seen whether the molecular organization takes the form of microfibrils as previously described^{1–9} or is related to some form of superlattice structure based on an underlying quasi-hexagonal lattice³⁷. Although the observed variability in the side spacing could be due to variable distortion of a single structural repeat, it could also be a result of viewing a superlattice structure along different lattice planes.

In order to clarify some of these points, we are carrying out a further analysis of longitudinal cryosections of tendon and we also hope to develop the transverse cryosectioning technique so that the transverse ultrastructure of collagen fibrils can be seen in two dimensions. In the mean time the early results reported here provide direct support for some form of intermediate molecular aggregate within collagen fibrils and they emphasize once again the power of the convolution camera in studying elusive features in the transverse structure of fibrous protein assemblies.

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Salt-dependent dynamic structure of poly(dG-dC) . poly(dG-dC)

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Until recently, all known nucleic acid duplex helices were thought to be conformationally related to A DNA or B DNA, these right-handed helices being characterized by C(3') *endo* or C(2') *endo* pucker for the respective sugar residues and by an antitiglycosidic bond¹. This situation has since drastically changed, for X-ray studies on crystals of alternating d(CpG) DNA fragments have demonstrated the existence of a left-handed double helix, termed Z DNA (ref. 2). In this novel double helix the base-pairing is of Watson–Crick type, but the cytidine and guanosine residues have the *anti* and *syn* conformations, respectively, and thus the repeating unit is two base pairs. Such a left-handed helix can also account for the X-ray diffraction pattern of alternating poly(dG-dC) . poly(dG-dC) fibres³. However, Pohl and Jovin have shown that in solution poly(dG-dC) . poly(dG-dC) undergoes a salt-induced cooperative transition⁴. It is very likely that the left-handed double helix is relevant to the high-salt conformation of poly(dG-dC) . poly(dG-dC) and that the low-salt conformation of poly(dG-dC) . poly(dG-dC) belongs to the B-DNA family^{3,5}. The techniques previously used to study the salt-induced transition of poly(dG-dC) . poly(dG-dC) in solution—circular dichroism (CD), absorption spectroscopy⁴ and nuclear magnetic resonance⁵—give essentially a static picture of the conformation of the molecule. Such information, although fundamental, is incomplete because double-helical nucleic acids in solution are known to be subjected to thermal fluctuations resulting in transient conformations with opened base pairs^{6–9}. A particularly useful probe of the dynamic aspects of nucleic acid structure is the tritium exchange technique largely developed by Englander and colleagues¹⁰. We have now used this technique to study the dynamic structure of poly(dG-dC) . poly(dG-dC) and have found that this varies depending on whether high or low salt conditions are used.

The exchange rate of the five protons involved in the hydrogen bonds between guanosine and cytosine residues in poly(dG-dC) . poly(dG-dC) were measured at two different ionic strengths (1 M NaClO₄ and 3 M NaClO₄). As Fig. 1 shows, there is dramatic difference between the two hydrogen exchange curves, the overall exchange in high salt conditions being much slower than in low salt conditions. In 1 M NaClO₄, four protons are measured and they all exchange with the same rate characterized by an exchange half-time of 6 min. Identical results were obtained by Teitelbaum and Englander for poly(rG) . poly(rC) in the same pH conditions⁷. These four protons were assigned to the exocyclic amino protons of the guanine and cytosine whereas the missing proton corresponds to the guanine imino proton, whose exchange rate is too fast to be measured by this technique⁷. In contrast, all five protons of the (GC) hydrogen bonds are measured in 3 M NaClO₄. The exchange of two of these protons is surprisingly slow, the exchange half-time being about 7 h. Subtraction of these protons from the total exchange curve reveals that the remaining three protons have the same exchange half-time of about 20 min (Fig. 1 inset). To rule out any possible effect of the difference in NaClO₄ concentration on the chemistry of the exchange process, we have studied the exchange in 2.5 M NaClO₄ of poly(dG-dC) . poly(dG-dC) trapped in its low-salt conformation (this is possible because the kinetics of the transition between high and low salt conformation is very slow, with a relaxation time of 2 h in 2.5 M NaClO₄ (ref. 3)). All the measured points fall on the exchange curve obtained in 1 M NaClO₄, showing clearly that there are no measurable salt effects on the chemistry of the exchange process.

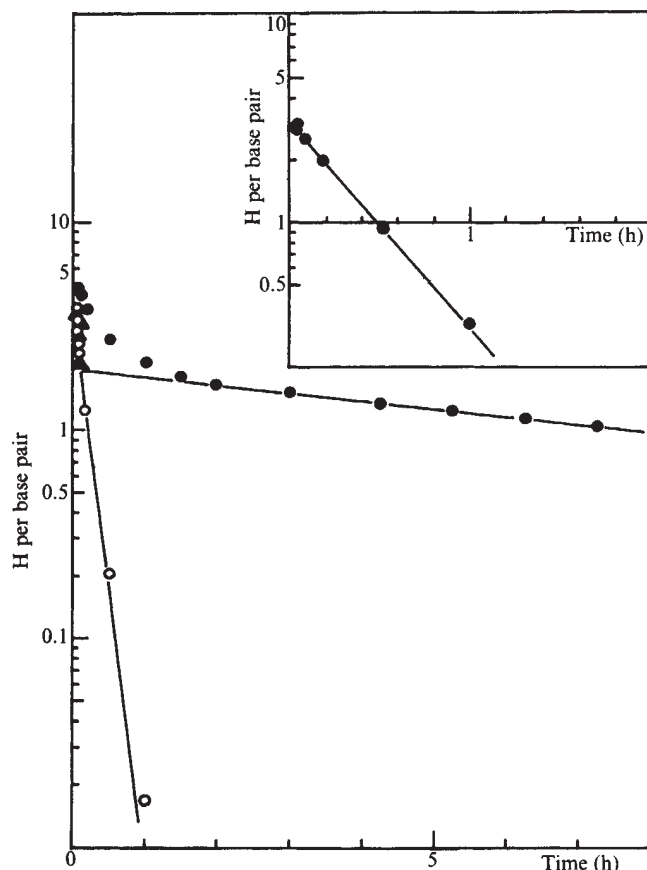


Fig. 1 Hydrogen-tritium exchange curves at 0°C for poly(dG-dC).poly(dG-dC) (PL-Biochemicals, lot 658-5) in 3 M NaClO₄ (●), 1 M NaClO₄ (○) and 2.5 M NaClO₄ (▲). The salt solutions were adjusted to pH 7.5 and phosphate buffer pH 7.5 then added to a final molarity of 10 mM. It was checked that the CD (circular dichroism) spectrum of poly(dG-dC).poly(dG-dC) in 1 M NaClO₄ was almost an inversion of the one in 3 M NaClO₄, in agreement with previous results⁴. The tritium Sephadex method of Englander was used¹⁰. All exchange experiments were carried out at 0°C. Exchangeable protons were labelled with tritium by incubating 0.5 ml of the nucleic acid solution (2 mg ml⁻¹) with tritiated water (10 μl, 1 Ci ml⁻¹) for 21 h. In all experiments the incubation solvent was identical to the exchange solvent except for the exchange experiment in 2.5 M NaClO₄, for which the incubation solvent was 1 M NaClO₄. To initiate the exchange of nucleic acid-bound tritium the incubation sample was passed through a Sephadex column (G-25 fine grade, 8 cm × 1 cm) previously equilibrated with the exchange buffer. This column removed the free tritium and the nucleic acid peak was collected in a test tube. To measure the amount of tritium still bound at any given time *t*, 300-μl aliquots were passed through a second Sephadex column (G-25 fine grade, 5 cm × 1 cm) previously equilibrated with the exchange buffer. This column removed the tritiated water formed during time *t*. The nucleic acid peak was collected in four test tubes which were analysed for radioactivity and nucleic acid concentration. The latter was determined by optical density at 260 nm, using a Zeiss PMQ II spectrometer and an extinction coefficient of 7 × 10³ and 6.3 × 10³ mol⁻¹ cm⁻¹ in 1 M and 3 M NaClO₄, respectively⁴. Tritium was measured in 0.5-ml aqueous samples with 6 ml of dioxan-based liquid scintillation mixture by counting in an Intertechnique SL 30 liquid scintillation counter. From the ratio of tritium counts per base pair, protons remaining unexchanged at *t* can be evaluated¹⁰. The gramme-atom concentration of protons in water was not corrected for the presence of NaClO₄ and was set equal to 111. For measurements of early time points (*t* < 5 min), a one-column procedure was used¹⁰. The exchange of the nucleic acid-bound tritium was initiated by passing 0.2 ml of incubation solution half-way down a column (8.5 cm × 1 cm). The flow was stopped to allow exchange to take place in the column during a suitable time interval; elution was then continued to complete separation of labelled nucleic acid and newly exchanged tritium. Fractions were collected and assayed as above. All necessary time corrections as indicated by Englander¹⁰ have been made.

It is not possible to decide whether the two protons are the cytosine or the guanine amino protons, although the latter are the more likely candidates. Indeed, X-ray crystal studies of d(GpC) oligonucleotides show that a hydrogen bond is formed between the guanine amino group and a water molecule which is also hydrogen bonded to a phosphate oxygen². Whatever the exact assignment of these five protons, our results show clearly

that the exchange rate of the protons is dependent on the conformation of poly(dG-dC).poly(dG-dC), which in turn means that the dynamic structure of the high and low salt conformations must be different.

Finally, we emphasize that this technique might be very useful for detecting the presence of the poly(dG-dC).poly(dG-dC) high-salt conformation in natural DNA. As already pointed out with respect to hydrogen exchange, the high-salt conformation is essentially characterized by the presence of two very slowly exchanging protons with a half-time at least 50 times greater than that measured for protons in double-stranded polynucleotides^{6,7}. The existence of these two protons together with the high sensitivity of the tritium-labelling technique should enable the presence of small amounts of high-salt poly(dG-dC).poly(dG-dC) conformation to be detected in natural DNA.

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Neutron diffraction identifies His 57 as the catalytic base in trypsin

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The mechanism of action of trypsin and other serine proteases has been widely debated¹⁻⁵, particularly with regard to the identification of the group at the active site which functions as the chemical base during the catalytic process. Attempts to resolve this question by a number of indirect techniques, including NMR, isotope exchange, difference IR and quantum mechanical calculations, have resulted in different identifications of this group^{6,7}. Neutron diffraction, because of its ability to locate hydrogen atoms experimentally, offers the most direct way of resolving this issue. Results are presented here from a 2.2-Å neutron data set for bovine trypsin covalently inhibited by a transition-state analogue, the monoisopropylphosphoryl (MIP) group. His 57 is clearly identified as the base in the catalytic process.

The catalytic site of the serine proteases is characterized by three invariant residues, a histidine (57), an aspartic acid (102) and a serine (195). The mechanism by which these enzymes hydrolyse peptide bonds can be described as a general base-catalysed nucleophilic attack on the carbonyl carbon of the substrate by the hydroxyl oxygen of Ser 195 (refs 8, 9). Coincident with this attack, the hydroxyl proton of the serine is transferred to the imidazole of His 57. The primary unresolved mechanistic question has been whether His 57 is the actual chemical base in the hydrolysis reaction (intermediate 2, Fig. 1) or whether the histidine acts as an intermediary through which Asp 102 functions as the base (intermediate 1). The neutron studies of myoglobin by Schoenborn and his co-workers¹⁰⁻¹², which have shown that well ordered hydrogen and deuterium atoms can be located in Fourier maps of approximately 2.0 Å resolution, suggested that neutron diffraction would be the most direct technique to resolve the question.

The trypsin used in this study was initially inhibited with diisopropylfluorophosphate, an inhibitor which specifically and

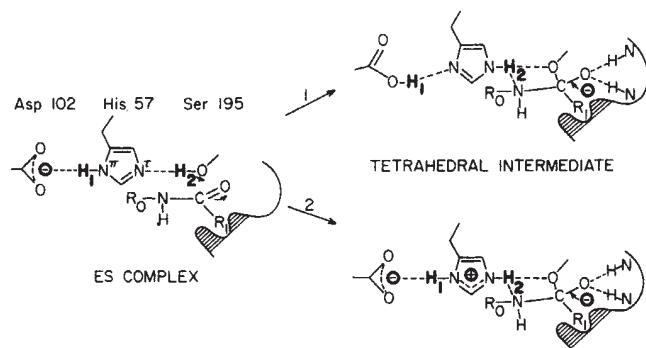


Fig. 1 The two different locations proposed for proton H-1 in the tetrahedral intermediate (TI) structure. In the formation of TI(1), H-1 shifts from N⁷ to Asp 102 (O-δ2) as proton H-2 transfers from the serine hydroxyl group to N⁷ of the imidazole. His 57 remains neutral throughout the reaction. In TI(2), proton H-1 remains on the imidazole as H-2 transfers. The imidazole becomes charged and forms a zwitterion with the carboxylate of Asp 102. The intermediate pictured here corresponds to that formed during acylation and differs from the deacylation intermediate (which MIP mimics) only by the replacement of the leaving group (R₀-NH₂) with a hydroxyl group.

covalently binds to the hydroxyl group of Ser 195 in serine proteases¹³. However, it was determined both chemically and crystallographically (R. M. Stroud, personal communication) that during the period of crystallization, this triester hydrolysed to the more stable diester form, producing MIP trypsin (Fig. 2). The geometry of this inhibitor closely resembles that of the tetrahedral intermediate¹⁴, which is believed to be the transition-state configuration for the hydrolytic reaction¹⁵⁻¹⁷. Analysis of this complex consequently allows the direct determination of the protonation states of the catalytically important residues at the crucial step in the hydrolysis.

Neutron data to 2.2 Å were collected at the single-crystal station at the Brookhaven High Flux Beam Reactor¹⁸. The

crystal of bovine trypsin was grown from MgSO₄ at pH 7 (ref. 14) and had a volume of 1.5 mm³. It was soaked for approximately 1 month in D₂O solution containing 8% MgSO₄ (pD 6.2, uncorrected) to replace most of the exchangeable hydrogen atoms with deuterium. This exchange greatly reduces the background scattering effects of hydrogen. In all, 8,700 independent reflections were collected using normal-beam rotation geometry. The initial phasing model was calculated by applying the appropriate neutron-scattering lengths¹⁹ to the refined trypsin X-ray coordinates of Chambers and Stroud²⁰. The first structure-factor calculation for the neutron data, excluding all hydrogen atoms and water molecules, gave an *R* of 0.304. The current model is refined to an *R* of 0.187. Table 1 summarizes the structure refinement statistics.

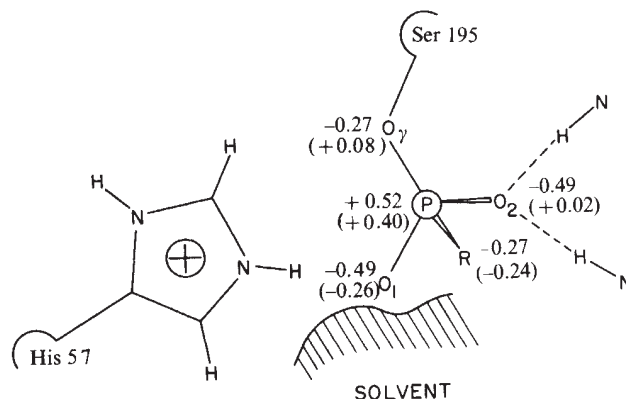


Fig. 2 Schematic diagram showing the orientation of the MIP group with respect to the imidazole of His 57. The MIP group resembles the deacylation intermediate of the reaction. In the real intermediate, the phosphorus is replaced by a carbon atom and oxygen O-1 by a hydroxyl group. Ligand R corresponds to the amino acid side chain, which in MIP is an isopropyl group. The individual charges on the atoms in the MIP group are given²⁵. The numbers in parentheses represent the computed differences in charge between corresponding atoms in the MIP group and the tetrahedral intermediate. The charges for the tetrahedral intermediate were obtained from a model reaction study (using methoxide ion and formamide) of the acylation step of the reaction²⁶. The charge on the hydroxyl group was assumed to be approximately the same as that on NH₂ in the methoxide-formamide intermediate. This assumption is supported by the calculated charge distributions of a hydroxyl-formamide intermediate²⁷. Electrostatic potential energies were calculated using the expression $E = \sum (q_i q_j) / (\epsilon_0 r)$. ϵ_0 , the dielectric constant, was 3.0; r is the distance between charges q_i and q_j .

Table 1 Refinement of monoisopropylphosphoryl trypsin

Prerefinement models		
Model	Source	Statistics
		$R = \frac{\sum F_o - F_c }{\sum F_o }$
Starting model*	Chambers-Stroud X-ray model ²⁰ with neutron scattering lengths ¹⁹	<i>R</i> = 0.304
Initial neutron model†	Hydrogen and deuterium atoms placed in model by inspection of difference maps	<i>R</i> = 0.255
Refinement	Approach	
Function		
Shifts in atom positions	Difference maps (curvature/gradient) ²³	
Reidealization of model coordinates and energy minimization	Model building and energy refinement ²⁴	
Current status‡	Nine cycles of refinement	<i>R</i> = 0.187

*Model contained no hydrogen or deuterium atoms.

† About three-quarters of the total hydrogen/deuterium atoms were located. 55 water molecules were also located and included in the phasing model.

‡ The current model is highly constrained, with r.m.s. deviations of bond lengths <0.015 Å and angles (bond and dihedral) <2.6° from the ideality. Nonbonded energy terms (van der Waals attraction-repulsion forces, electrostatic and torsional energies) have been refined.

As a major objective was to identify conclusively the group acting as the base, it was necessary to ascertain that the identification of the proton positions in the catalytic site was unambiguous. Three methods were used to test the preferred location of proton H-1 (Fig. 1). In method 1, the deuterium (proton H-1 was exchanged for deuterium during the soaking procedure) was omitted from the model and the full structure refined through one cycle of coordinate shifts and model reidealization. This refinement cycle was run to eliminate any bias in the phases that might have been introduced by a previous placement of this atom in the model. As seen in Fig. 3a, the difference synthesis calculated from these structure factors shows a distinct preference for the deuterium to reside on the imidazole.

In method 2, a deuterium atom was placed by stereochemistry on the π nitrogen of the imidazole and its position refined. After two cycles, the deuterium exhibited stable refinement characteristics and a relatively low temperature factor, indicating that it was tightly bound to the parent N⁷ atom. This process was then repeated with the deuterium placed in the proposed alternative position on the O-δ2 of the Asp 102 side chain. In this case, the refinement was quite unstable, and the deuterium atom

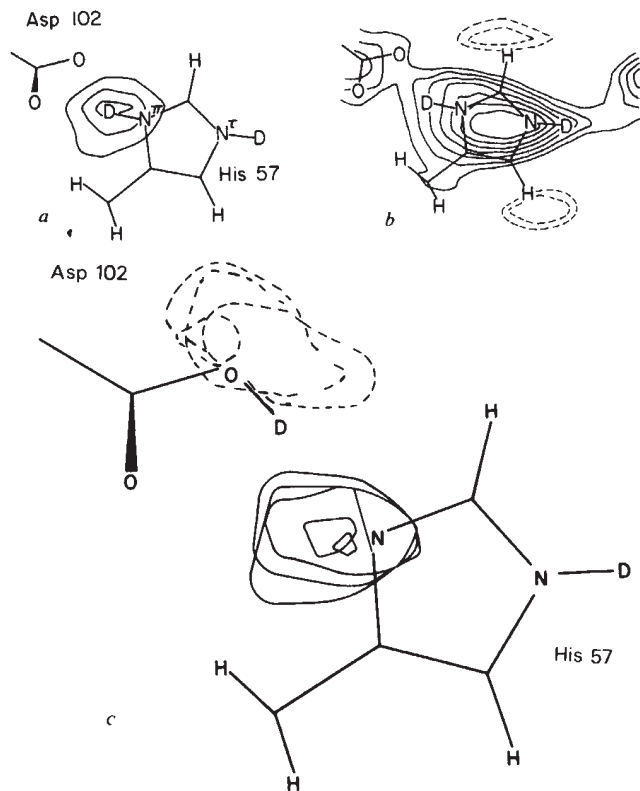


Fig. 3 *a*, A difference map $((F_o - F_c) \exp(i\phi_c))$ calculated with only the deuterium (H-1) between the His 57 and Asp 102 side chains left out of the phases. The difference peak is approximately 5.5σ above the background level and shows the deuterium to be bound to the imidazole nitrogen. *b*, A Fourier map computed with terms $(2F_o - F_c) \exp(i\phi_o)$. In this map, both of the catalytically important deuteriums (corresponding to H-1 and H-2 in Fig. 1) were omitted from the phases. This map clearly shows that both are located on the imidazole. (Hydrogen atoms appear as negative density because of their negative neutron-scattering length. The peaks for the ring hydrogens seem to be displaced from their true positions due to the limited resolution of the map. At $2.2 \text{ \AA}$, a portion of the negative density peak of a ring hydrogen overlaps the positive peak of the parent atom, effectively cancelling density between the atoms and giving the illusion that the peak has been translated.) *c*, A difference map in which the deuterium was placed by stereochemistry on atom O-δ2 of Asp 102 (if there were a proton coordinated to Asp 102, it would be on O-δ2). The difference density peak clearly indicates that the preferred location of the deuterium is on the imidazole of His 57.

resided along a distinct gradient, giving a clear indication of misplacement.

Method 3 involved the qualitative examination of two independent difference maps. For the first map, structure factors were calculated with the deuterium atom on the N^π of His 57. In agreement with the refinement results, a nearly featureless map was produced, signifying that this placement was in close accord with the data. In contrast, placement of the deuterium on the O-δ2 of Asp 102 resulted in the interpretable difference map shown in Fig. 3c. The difference peak clearly indicates that a substantial shift towards the imidazole ring is warranted.

The results from each of these three techniques independently support the conclusion that the mechanistically important proton is coordinated to the imidazole of His 57. Taken together, we believe they offer compelling evidence that, at physiological pH, the protonated species in the tetrahedral intermediate is the imidazole of His 57 rather than the carboxylate of Asp 102.

To eliminate any possibility that the protonation states of the aspartate and histidine observed in this neutron diffraction study were artefacts due to the substitution of a MIP group for a real tetrahedral intermediate, a series of calculations was performed

to establish that the phosphate ester in trypsin also models the structure of the tetrahedral intermediate with respect to the location of the critical atom (H-1 in Fig. 1). Although both the phosphate ester (MIP) and the tetrahedral intermediate (TI) possess a net charge of -1 , the centres of their charge distributions, with respect to the centre of the positively charged imidazole, differ somewhat ($R_{\text{MIP}} = 4.3 \text{ \AA}$, $R_{\text{TI}} = 4.6 \text{ \AA}$). However, energy calculations suggest that this difference of $0.3 \text{ \AA}$ in the centres of the distributions is unlikely to be large enough for the MIP group to promote an interchange of the protonation states of His 57 and Asp 102 (from TI(1) to TI(2) in Fig. 1), as the calculated electrostatic potential energy (Fig. 2) derived from the interaction between the imidazole and the MIP group differs by only $\sim 6\%$ from that obtained with a model of the expected tetrahedral intermediate.

Possible changes in the pK_a of His 57 were also explored by the method of Gurd *et al.*^{21,22}. Assuming no attenuation due to solvation, it was estimated that the MIP group can increase the pK_a of His 57 by, at most, 0.8 pK units, whereas the tetrahedral intermediate was estimated to increase it by about 0.6 pK units. In addition, the phosphoryl oxygen adjacent to the imidazole (O-1, Fig. 2), the largest contributor to the calculated pK effect, is solvent accessible; therefore, the influence of this oxygen should be significantly attenuated by solvation. If the assignment of pK 's by Hunkapiller *et al.*² were correct, the pK_a of His 57 would have to be raised by about 3.0 pK units to interchange the protonation states. As the calculated electrostatic effects due to the MIP group are much too small to accomplish this, it can be stated with confidence that the protonation states observed in the neutron diffraction experiment correspond to those in a true transition state.

The implications of the neutron diffraction results for the understanding of the mechanism of action of the serine proteases are twofold: (1) Those mechanisms which assigned Asp 102 as the base can now be eliminated from consideration and (2) the conclusive assignment of His 57 as the base will allow the major experimental emphasis of future work to focus on other important aspects of the mechanism. A more detailed description of the crystallographic results will be published elsewhere.

This research was carried out at Brookhaven National Laboratory under the auspices of the US Department of Energy. We thank Drs B. Schoenborn and J. Cain, who designed and developed the crystallographic station at the Brookhaven High Flux Beam Reactor, for their help and suggestions, Dr R. Stroud for donating the trypsin crystal and Dr J. Hanson for help with the computer graphics.

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BOOK REVIEWS

Archaeologist extraordinary

Gordon R. Willey

V. GORDON Childe (1892–1957), an Australian by birth, who became, successively, the Abercromby Professor of Archaeology at the University of Edinburgh and the Director of the Institute of Archaeology at the University of London, was the foremost prehistoric archaeologist of his time. His influence on the archaeological profession, both in English-speaking countries and elsewhere, was profound and continues to be felt; and his best-known writings, although by no means “popular” in an ordinary sense, have been read in circles far beyond those of the profession.

Numerous articles, reviews and commentaries about Childe and his work have appeared over the years, but this study by Professor Bruce G. Trigger, of McGill University, is the first book-length intellectual and professional biography of this extraordinary man and scholar. Trigger is well-prepared for and suited to the task. As a Canadian he is familiar with both American and Old World archaeological histories, and his own research has spanned both archaeology and ethnology.

The biography begins with a brief consideration of Childe the controversial figure — “Man and Myth”, as Trigger terms it — the conceptions and misconceptions about Childe that have developed in the archaeological community since the 1930s and 1940s and the publication of his two most widely read books, *Man Makes Himself* (Watts, 1936) and *What Happened in History* (Penguin, 1942). Trigger follows this with a review of European archaeology and archaeological thinking before Childe and indicates just how the latter both learned from and began to change this thinking. Subsequent chapters carry the story forward in time, although as the author’s primary intention is to trace Childe’s intellectual development there are, of necessity, many retrospective and anticipatory observations in his skilful analysis.

In such early works as *The Dawn of European Civilization* (Kegan Paul, 1925) Childe built on the artifact typologies and synchronisms of his predecessors, but he did it better, presenting the most complete and satisfactory synthesis of European prehistory up to that time. He accepted Kossinna’s “archaeological culture” concept as a useful working device but rejected its racist grounding; while he abjured rigid unilinear evolutionism he

Gordon Childe: Revolutions in Archaeology. By Bruce G. Trigger. Pp.207. (Thames and Hudson/Columbia University Press: 1980.) £10, \$22.50.



Gordon Childe: “the foremost prehistoric archaeologist of his time”.

then began his life-long attempt to develop a more sophisticated set of cultural evolutionary theories, doing so in conjunction with diffusionist thinking more akin to that of Montelius than to the extravagances of Eliot-Smith and Perry. This was followed up directly in the 1920s and 1930s with those great books, *The Danube in Prehistory* (Oxford University Press, 1929), *The Most Ancient East* (Kegan Paul, 1928), *The Bronze Age* (Cambridge University Press, 1930) and *New Light on the Most Ancient East* (Kegan Paul, 1934), all of which trace the course of development of Neolithic life and subsequent civilization from the Near and Middle East into Europe. In doing this, Childe’s consideration of such processes of civilization as the stabilization of institutions, the widening of trade horizons, the capitalization of surplus wealth and the consolidation of smaller communities into urban settlements clearly foreshadowed present-day archaeological concerns. The same is also true of Childe’s pioneer

settlement pattern studies in Scottish archaeology. Indeed, the last sentence of Trigger’s biography sums this up forcefully and succinctly:

It is surely a measure of Childe’s intellectual stature that, in spite of the buffeting of two decades, the major questions that he dealt with at the end of his career are now more critical to the future of archaeology than they were when he first raised them.

Here Trigger is referring primarily to some of Childe’s later, more explicitly theoretical works of the 1940s and 1950s; but even in these earlier writings which were addressed to the data contexts of prehistory it is obvious that Childe had a sense of problem that overreached that of most of his contemporaries.

Childe is, of course, best known as a theoretician, and, cognizant as he was of the changing nature of the archaeological record, it was his own opinion that it was in this sphere that he would be best remembered; nevertheless, it cannot be overstressed that Childe was “data bound” in the very best sense of that term. This comes out, again and again, in all of his publications, as Trigger has demonstrated so tellingly. He was committed to the idea that the archaeologist should be concerned with the explaining of society, human behaviour and ideas, but he did not believe that this could be done successfully and satisfactorily out of the context of the facts of prehistory and history.

Much has been made of Childe as a Marxist thinker; but as Trigger shows us in his chapters “Human Progress and Decline”, “Archaeology and Scientific History” and “Societal Archaeology”, Childe was highly ambivalent about Marxist doctrine. Marxist dialectics presented to him a way of thinking, but there was always a tension, a conflict within himself as he measured the realities of the archaeological data in and on the ground with Marxist doctrines. In doing so he was cautious, critical, even pessimistic. He was a materialist but he was also a humanist. Out of these complexities and contradictions came the most creative archaeological writing of the mid-twentieth century.

No treatment of Childe would be, or could be, complete without some reference to the “new archaeology” of the 1960s and 1970s, and Trigger has provided this in a final chapter, “Beyond the New Archaeo-

logy". While Childe was not an immediate intellectual prototype for it, it cannot be denied that his writings have helped prepare the way. Childe was ever interested in the search for regularities, "laws" in human cultural and social behaviour. Trigger makes the point, however, that Childe differed from the "new archaeologists" in seeing human behaviour as changing, not immutable. In this he was Marxist for he believed that behaviour changed as social context changed. For him the search for "laws" was a legitimate but not the primary objective of archaeology. The primary objective and the future of archaeology lay, in his mind, with history — in the full context of social and cultural history. With this conviction, as Trigger says, he would have had little sympathy with archaeological examinations of "fragmentary" behaviour, behaviour out of context.

It is redundant or over-obvious to say that archaeology is "as large as life" — but, indeed, it is; or at least it may legitimately encompass all aspects of human life in the past. Gordon Childe was very much aware of this. Those who view him as a special pleader or partisan adherent of limited theoretical views overlook the facts of his devotion to and masterly control of the data, the traditional data of form, time and space. What he did was to ask new questions and in attempting to answer these questions — all of which were of importance in understanding mankind's past — he made us aware of how much of the potential record remained to be discovered. This was disturbing — and exciting — but it raised the discipline of archaeology to a whole new level of consciousness. □

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Red bed diversity

J.R.L. Allen

Continental Red Beds. Developments in Sedimentology, Vol. 29. By P. Turner. Pp. 562. (Elsevier Scientific: 1980.) Dfl. 145, \$70.75.

THIS is an important and original book in which the author sets out to synthesize and review what we currently know of the nature, depositional environments and processes, and diagenesis of continental red beds. Continental red beds and the "red-bed problem" have fascinated sedimentary geologists for many years, and in the minds of some investigators have seemed of particular petrogenetic significance. Dr Turner's book illustrates the variety of the issues raised by investigation of continental red beds and shows that it is

naïve to conceive of a single red-bed problem. Its value is rather to illustrate the remarkable diversity of continental red beds, a diversity of diagenetic environments and history as much as of depositional environment.

There are four sections in the book. The first considers the tectonic and climatic setting of ancient red beds and leads to a preliminary assessment of the question of the origin of red coloration. A practical classification of red beds is advanced, based on depositional environment, colour characteristics and diagenesis. The second and longest section is a thorough review of the depositional processes and environments of modern continental sediments and of ancient reddened counterparts, such as the European Lower Permian, the Upper Coal Measures of South Wales, and the Old Red Sandstone of the British Isles. The third section is concerned with the diagenesis, mineralogy and geochemistry of continental red beds. The fourth comprises two chapters, of which the first is a general account of the magnetization of continental red beds, and the second a study of magnetization case-histories (Proterozoic basins of western Canada,

Late Precambrian of Scotland, Moenkopi Formation of western USA, late Cenozoic red beds of Baja California). These clearly illustrate the complex controls on the magnetization histories of continental red beds and the difficulties of interpreting palaeomagnetic data, even when a wide variety of supporting field, petrographic and geochemical information is available.

Dr Turner's book is a carefully researched and well-written account of the sedimentology of continental red beds, the author moving confidently from large-scale phenomena to problems on the smallest of scales. The book was prepared from camera-ready typescript (there are a few small errors) and is well-illustrated by line drawings and photographs. There is an extensive and up to date list of references. The book is expensive but no sedimentologist concerned with continental sediments can afford to neglect it. □

J.R.L. Allen is Professor of Geology at the University of Reading. His research interests include the sedimentology of the Old Red Sandstone (a series of continental red beds of Devonian age) in southern Britain.

Introduction to liposome research

Timothy D. Heath

Liposomes in Biological Systems. Edited by G. Gregoriadis and A. C. Allison. Pp. 424. (Wiley: 1980.) £23, \$69.

Liposomes in Biological Systems is the first book to be devoted completely to this aspect of liposomes. Its publication reflects the considerable expansion which has recently occurred in liposome research.

The book begins with a contribution by A.D. Bangham who traces the history of liposomes as he and others first characterized them, and their subsequent use by biologists. Following this, Dr Gregoriadis summarizes the potential applications of liposomes as drug carriers, and expresses his hopes for the future.

In the subsequent chapters various specialist authors concentrate largely on their own contributions to the field. J.H. Fendler describes the various factors concerned with encapsulation of drugs. G. Poste gives a critical review of liposome-cell interactions, which will enlighten those who may still believe the predominant mode of vesicle-cell interaction to be fusion. This area of liposome research in particular has been the subject of much recent reconsideration. Several chapters are devoted mainly to the *in vivo* fate of liposomes and their contents, including the uptake of enzyme-bearing liposomes by G. Weissmann and M. Finkelstein. G. Scherphof *et al.* have described their efforts to elucidate the interaction of

liposomes with serum components, and H. Kimmelberg discusses his work on the *in vivo* fate of encapsulated methotrexate. I. H. Shaw and J. T. Dingle describe the interarticular injection of steroid-containing liposomes which is indeed one of the most promising applications of liposomes so far developed. (It would also have been appropriate had this volume included a chapter on the work of C. D. V. Black, R. R. C. New and C. R. Alving on the use of liposome-encapsulated antimonials in the treatment of visceral Leishmania, which clearly may be one of the first successful therapeutic applications of liposomes.) The final specialist chapter, by G. M. K. Humphries, covers the use of liposomes for studying *in vitro* immune reactions, which will undoubtedly become a major area of liposome research.

The contents of this volume are very similar to those of Vol. 308 of the *Annals of the New York Academy of Sciences* (1978), and most of the contributions appear to have been written between 1976 and 1978. The delay in publication is unfortunate, although Dr Gregoriadis has added a short chapter which summarizes much of the more recent literature.

In conclusion, this volume will provide a useful introduction for those unfamiliar with the field. □

Timothy D. Heath is a research fellow at the Cancer Research Institute, University of California, San Francisco.

Telescopes of the future: heading for the supergiants

V.C. Reddish

Optical and Infrared Telescopes for the 1990s. Edited by Adelaide Hewitt. Two volumes, pp.1260. (Kitt Peak Observatory: Tucson, Arizona, 1980.) \$35 + \$15 airmail.

AT A time when the financial outlook for fundamental science may appear bleak, these two volumes should raise the spirits even of the pessimistic. Undaunted, more than 200 scientists and engineers gathered at Tucson, Arizona, in January 1980 to discuss how best to apply the latest technologies to the design and construction of very large optical and infrared telescopes, and what further technological research and development is needed for that purpose in the immediate future. This record of their deliberations makes exciting reading.

A wide range of possibilities was considered for telescopes with apertures mostly in the range 7m to 30m; the pros and cons of monolithic versus segmented mirrors, multi-mirror versus multi-telescope, single telescope versus arrays, were argued. Everyone, it seems, accepts that mirrors will be thin whatever configurations are adopted; the UK Infrared Telescope has proved that they can give optical performance and major cost savings.

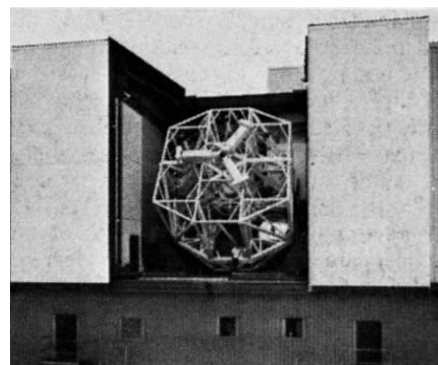
Although various kinds of mountings were mentioned — alt-az, inverted alt-az, siderostat, steerable dish — not a lot of consideration was given to this aspect of telescope construction, and even less to the problems of domes and buildings. These are areas where major cost savings may be made and it could be worthwhile having a follow-up conference on the topic of housing very large telescopes. A radical breakaway from historical approaches (even recent ones such as the MMT building and the CERGA concept) may be possible and desirable if large economies are to be made, sufficient to make the construction of very large telescopes acceptable propositions. Do we really need to erect large concrete towers with rotating domes on top, or even rotating buildings, to protect the telescope from the weather and to provide a satisfactory working environment for engineers when it is not being used, and to shield it from the wind when it is? The cost is high not only in financial terms but in the deleterious effect which the building usually has on the astronomical seeing. I think that far more satisfactory structures could be made at costs one to two orders of magnitude less than those current.

A heartening feature of the discussions, however, was the recognition of the need to pay attention to the relationship between the design and aperture of the telescope, and the effects of the atmosphere on the image. These are of course functions of the

wavelengths at which the telescope is to be used and therefore relate to the astronomical research objectives and to the instrumentation — spectrometers and detectors — to be employed. This chain forms a recurring theme throughout the discussions; repeated attention was drawn to astronomical objectives and to the need to make the whole telescope system efficient. Considerable further advances in computer control are possible and substantial gains in efficiency can result from more extensive use of photoelectric autoguiders. It is surprising, as one participant pointed out, that autoguiders are still so little used. Every telescope built by the Royal Observatory Edinburgh since 1962 has been autoguided and that has been a major factor in the high quality of photographs produced by the UK Schmidt Telescope in Australia which was equipped with one from the outset; if it can be done for a Schmidt telescope, it can be done for any telescope.

There were so many contributions that it is impossible to mention more than a few. The experimental data on deformable mirrors by J.W. Hardy I found fascinating; also the discussion of (iso)thermally invariant structures by F.D. Drake and the paper on imaging by C.H. Townes. The most important technological papers, however, were probably those concerned with the matter stated succinctly by T.S. Mast and J.E. Nelson:

The central problem with a segmented mirror is to assemble the small mirrors and maintain their orientations and positions so they form the figure of a single large optical quality mirror.



A telescope of today: the multi-mirror telescope on Mount Hopkins, Arizona.

Much effort, study and experiment will be devoted to this problem in many observatories around the world during this decade. It will be a happily competitive business, a race to be the first with the biggest and the best. It is difficult not to believe that the problem will be solved and the 1990s will see giant telescopes with apertures of 25m or more probing the outer half, the earlier half, of the Universe, intent upon discovering its structure, chemistry and evolution. The prizes in new knowledge in one of the most fundamental of our sciences could be immense, a just and proper reward for the supergiant telescope builders.

V. C. Reddish was until recently Director of the Royal Observatory Edinburgh and Professor of Astronomy in Edinburgh University. The UK Schmidt Telescope in Australia, the UK Infrared Telescope in Hawaii and the COSMOS measuring machine at Edinburgh were built under his direction.

Between the sheets

J.M. Thomas

Intercalated Layered Materials. Edited by F. A. Levy. Pp.562. (Reidel: 1979.) Dfl.150, \$78.

WHEN a sheet-like structure assimilates guest species into its interlamellar spaces the phenomenon is known as intercalation. The uptake of ammonia by clay minerals in soil, first identified in 1850, is an example of such a process, so also is the swelling of clay when immersed in water, an effect known to the potters and ceramicists of antiquity. When transition-metal chalcogenides (such as niobium diselenide or molybdenum disulphide) take up alkali metals, the intercalation may dramatically alter the electronic properties of the parent solid: semiconductors become metals and metals semiconductors, purely as a con-

sequence of the donation of electrons into empty or partially filled bands. Moreover, the addition of a wide range of organic materials (amines, amides, acids, pyrimidines and numerous heterocyclics and their derivatives) changes the superconducting transition temperature of the chalcogenide. When graphite, the best known and most widely studied of layered solids, assimilates certain electron-donating or electron-accepting guests, the process is accompanied by many significant structural, chemical and electronic changes. Alchemists would doubtless have been aroused to discover that when graphite is exposed to the vapour of potassium the glistening black solid is "transmuted" into a specular, golden mass. Intercalated species generally carry a residual charge and they usually take up

well-defined positions in directions parallel and perpendicular to the sheets. For graphite, but not, in general, for all other layered hosts, there is also the phenomenon of staging: the guests enter only some of the available interlamellar spaces and in an ordered fashion. The resulting range of structural possibilities is bewildering.

In this, the sixth volume in a worthwhile series devoted to the physics and chemistry of materials with layered structures, there are some outstanding chapters which elegantly combine both comprehensive coverage and critical assessment. All of the chapters are, to a greater or lesser degree, useful, but one or two lapse into the kind of language which scientists, in their quests for funding, inflict upon grant-awarding committees. Thus we read "... graphite intercalation compounds provide a near-ideal training vehicle in electronic materials science ... The frequent observation of surprising or dramatic results provides a beneficial stimulating effect, and the ever-present potential for gross error instills [*sic*] a healthy caution". Such sentiments could equally justify a reinterpretation of the Egyptian Book of the Dead or the factors that led to the emergence of Sufism.

Solid-state physicists, chemists and materials scientists will find much that is stimulating and rewarding in this monograph. I know of no more comprehensive survey of the intercalates of transition metal chalcogenide, MX_2 , than that provided here by G. V. Subba Rao and Shafer, and no superior summary of the exciting developments that have occurred in studies of graphite intercalates since they were discovered by Schaufhault in 1841 than the chapter by Herold. The former deals admirably with, *inter alia*, the analogy between "solvated" intercalates (such as $Na^+(\text{sol.})_y \cdot (MX_2)^{-}$ where solv. can be water, formamide, glycol, ethers, amines or amine oxides) and classical polyelectrolytes, well-known to colloid scientists. The latter covers all relevant aspects of graphite intercalate including two tentative schematic models for "interpenetrating stages" and "residue compounds" (where guest species are well and truly enclathrated up to quite high temperatures), the correctness of each of which has recently been confirmed by electron microscopic studies.

Ubbelohde's opening chapter summarizes his own well-known contributions to graphite intercalates, which receive further attention in this volume by M. S. and G. Dresselhaus, who review lattice mode structures explored principally by the Raman effect, and by Fischer, who assesses their electronic properties. Other chapters, devoted to chalcogenides, include an informative, closely argued contribution by Acrivos on the actual process of intercalation (its kinetic, mechanistic and thermodynamic aspects as well as likely bonding modes); a neat appraisal, by Beal, of the transition-metal intercalates of Group VA dichalco-

genides; a similar account by Rouxel of alkali-metal intercalates of hosts such as MX_2 , MX_3 and MX_4 ; and a rather telegraphic account by Samoana and Wollam of some intercalates of MoS_2 .

The final chapter, entitled "Applications of Intercalation Compounds", by Whittingham and Ebert, though it covers thoroughly the electrochemistry of the various intercalates discussed elsewhere in this volume and touches upon the utility of variants of beta alumina as solid electrolytes and on Li/TiS_2 as typical, reversible, solid cathode material for commercially viable "solid-state" batteries, is rather brief in its attention to other chemical applications. True, when this book was written, which I estimate to be nearly three years prior to publication, not a great deal had been unearthed from the laboratory (or from existing literature!) about the range of chemical reactions that are "catalyzed" by various intercalates (for example the Fischer-Tropsch reaction and the ammonia synthesis). But it seems odd that, apart from the very last sentence in the book, there is no mention of one of the largest families of intercalates yet discovered — those formed by kaolinite, montmorillonite, hectorite and vermiculite. These so-called kandites and smectites form more or less stable intercalates with a wide range of polar and non-polar

molecules (including alcohols, hydrocarbons, amines, amino acids, ketones, purines and so on). Although the structures of these insulating, clay-based intercalates, are, with a few notable exceptions, no better elucidated than the structures of many of the intercalates discussed in this volume, what is abundantly clear is that the range of highly selective organic chemical conversions — dimerizations, etherification, lactonization, hydrogen transfer, ester and amine syntheses — that may be effected through their agency is already impressive and continues to grow. Recently, Weiss and others demonstrated that intercalated amino acids may be stimulated to produce peptides inside a sheet silicate, thus vindicating Bernal's well-known speculation about the role of clay minerals in the origin of life. Reflecting on these thoughts, one recalls that in the Biblical account of Creation the Hebrew verb "to form" in Genesis 2:7 is also the technical word used for the potter forming the clay into a vessel.

This book can be unreservedly recommended, despite a few minor errors such as the omission of many of the references given in the main text from the index of names.

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The biology of potassium

Clive Ellory

Cell Potassium. By Roderick P. Kernan. Pp.200. (Wiley-Interscience: 1980.) £17.30, \$40.65.

CONTROL of potassium, the dominant intracellular cation, is of fundamental interest and an obvious topic for a monograph. Appearing 15 years after its modest ancestor, *Cell K* (Butterworths, 1965), *Cell Potassium* is a more authoritative work, increased in breadth and depth. The author has retained the general framework, and certain figures and anecdotes are familiar (including the curiously obscure story about the *Discovery's* dredge of sharks' teeth). Plants and geology are discussed first, followed by a useful chapter on measuring techniques, which surprisingly omits any reference to micro-probe analysis. The author is clearly at his happiest dealing with nerve and muscle, providing a good account of anomalous rectification, action potentials and gating currents. The sodium pump is treated competently, at a level high enough to include the occluded K form and conformational states, but without some of the more byzantine complexities of this system (and, thank goodness, no mention of vanadate). LK cells receive flatteringly extensive treatment, which makes the omission of KCl co-transport in avian

erythrocytes and Ehrlich cells more surprising. Similarly, the Gardos effect gets short shrift.

Mitochondrial K movements and the chemiosmotic hypothesis are adequately covered, although a basic revision to refresh the amateur's memory on mitochondrial structure would be of value. This comment applies to other sections of the book; to some extent it appears to be addressed to the already informed, in that a working knowledge of the specific subject being covered is often assumed.

A short chapter is devoted to the important yet neglected role of K as a co-factor in many biochemical systems, before the book closes with a consideration of K in epithelial transport. Extended treatment of the Koefoed-Johnsen-Ussing model is tempered by discussion of other interesting epithelia like that of the cochlea, but the final brief chapter on interactions between body K and pH sits rather uneasily.

Nevertheless this is a scholarly work which, in the most fluent chapters, on excitable tissues, sets a high standard. With its unique thematic approach, it should serve as a useful reference work.

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